TISSUE TRANSGLUTAMINASE AND ITS EFFECTS ON CELL MIGRATION AND SURVIVAL

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2013

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Cornell University 2013

Tissue transglutaminase (tTG) is a GTPase and acyl transferase which catalyzes the formation of covalent crosslinks between two protein substrates. tTG expression and activation are frequently up-regulated in different types of human cancer, where it has been shown to be important for enhancing cell motility. Using HeLa cervical carcinoma cells as a model system, I show that a membrane-associated pool of tTG becomes activated and re-distributes to the leading edges of migrating cells upon EGF stimulation. Immunoprecipitations of tTG from the membrane fractions of EGF-treated HeLa cells led to the discovery that tTG binds the heat shock protein (Hsp)70 family of molecular chaperones. tTG and Hsp70 co-localize at leading edges, and this localization is dependent on the ATP hydrolytic activity of Hsp70, as inhibitors against this function prevent both tTG and Hsp70 from localizing to leading edges. More importantly, these inhibitors also block the EGF-dependent migration of HeLa cells and the constitutive migration of MDA-MB231 cells, suggesting that Hsp70 helps localize tTG to leading edges to facilitate its role in promoting cell migration.

While tTG has been shown to influence a number of aspects of cancer progression, to what degree tTG works with oncogenic proteins to elicit these outcomes versus its intrinsic ability to impact malignant transformation is unknown. Thus, I have examined how ectopic expression of tTG in a normal (non-transformed) cellular context influences the behavior of

these cells. Using NIH3T3 fibroblasts stably expressing the vector alone or a Myc-tagged form of wild-type tTG, I found that tTG strongly protected these cells from serum-starvation-induced apoptosis by activating the PI3-kinase/mTOR/p70 S6-kinase pathway. tTG binds c-Src and PI3-kinase, and the formation of this complex is critical for the activation of the PI3-kinase signaling pathway. Activation of PI3-kinase signaling is essential for tTG's ability to promote cell survival, as inhibition of any component in this pathway, including Src, PI3-kinase, or mTOR, eliminates the protective effect afforded to the cells by tTG expression.

BIOGRAPHICAL SKETCH

Lindsey Boroughs was born and raised in Jackson Springs, North Carolina and was the youngest of three daughters. Upon graduating from Pinecrest High School in 2005, Lindsey attended the University of North Carolina–Wilmington and decided to direct her studies toward her two favorite subjects, Science and Mathematics. During her last year at UNC-Wilmington, Lindsey completed an Honors Thesis, performing biochemistry research in the laboratory of Dr. Christopher Halkides, where she worked on generating a stable analog of the phosphorylated form of CheY, a bacterial signaling protein, for x-ray crystallographic studies. With encouragement from her professors, Lindsey applied to graduate school and was accepted at Cornell University. Lindsey graduated from UNC-Wilmington *summa cum laude* with honors with a B.S. in Chemistry (concentration in Biochemistry) and a minor in Mathematics.

After coming to Cornell University in 2008, Lindsey became interested in cancer research and joined the laboratory of Dr. Richard Cerione. She transitioned into cell biology research, studying a novel GTP-binding protein known as tissue transglutaminase (tTG) whose expression and activation are frequently up-regulated in human cancer. Her studies focused on learning more about how tTG contributes to cancer progression. The years she spent studying signal transduction pathways and their relationship to cancer furthered her desire to continue in this field with the ultimate goal of designing better therapies to target this disease.

Upon completing her Ph.D., Lindsey plans to begin postdoctoral work in the laboratory of Dr. Ralph DeBerardinis at the University of Texas Southwestern Medical Center. The research in this lab is focused on understanding how cancer cells reprogram their metabolism to

meet increasing proliferative demands with the goal of designing therapies to target these tumor cells based on their metabolic signatures.

In memory of Matt Houston Barnes -

for reminding me to live life to the fullest and giving me the courage to follow my dreams.

ACKNOWLEDGEMENTS

First, I would like to thank my family for their love and support throughout my life. They always allowed me to make my own decisions and encouraged me to follow my heart. They made me believe that my best was always good enough and that there was nothing I could not achieve. I am truly blessed to have them in my life.

I have always believed that everything in life happens for a reason, and coming to Cornell and joining the Cerione lab was no exception. I am extremely grateful to Dr. Cerione for allowing me to be a part of his lab. He taught me how to think about science and set the best example of how to be a successful and inspiring lecturer. But more importantly, he created an enjoyable atmosphere in the lab which made it easy to want to come to work. It will be hard for me to ever find a place that feels as much like "home" as this one does.

I also want to thank Dr. Marc Antonyak. I could not have asked for a better mentor. He patiently helped me throughout these past few years – from teaching me how to do the experiments, to discussing data, to reading my papers and grants helping me become a better writer. He was instrumental in helping me develop as a researcher and for that, I cannot thank him enough.

I would like to thank my committee members, Dr. Barbara Baird and Dr. Ruth Collins, as well as Dr. Maurine Linder, for their support and guidance throughout my graduate career.

I was also blessed have great labmates and friends, "Team TG" – Bo Li, Jingwen Zhang, and Garima Singh, as well as Jared Johnson, Laura Desrochers, Bridget Kreger, Kai Su Green, Joe Druso, Joy Lin, Shawn Milano, Michael Lukey, Sungsoo Yoo, and Clint Stalnecker, and the

rest of the Cerione Lab. And a special thank you to my dearest classmates and friends – Maxim Kostylev, Patrice Ohouo, Ella Chang, and Leila Toulabi for being there for me throughout the past few years.

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LIST OF ABBREVIATIONS

AD: Alzheimer's disease

BPA: biotinylated pentylamine

BSA: bovine serum albumin

CS: calf serum

DAPI: 6-diamidino-2-phenylindole

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EMT: epithelial-to-mesenchymal transition

ERK: extracellular signal-regulated kinase

FBS: fetal bovine serum

G-proteins: guanine nucleotide-binding proteins

GAP: GTPase activating protein

GDP: guanine diphosphate

GEF: guanine nucleotide exchange factor

GTP: guanine triphosphate

HD: Huntington's disease

HIF1α: hypoxia-inducible factor 1 alpha

HPR: N-(4-hydroxyphenol)retinamide

HSP70: heat shock protein 70

IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IKK: IκB kinase

JNK: c-Jun N-terminal kinase

MAPK: mitogen activated protein kinase

MDC: monodansylcadaverine

mTOR: mammalian target of rapamycin

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

OCIC: ovarian cancer initiating cells

p70S6K: p70 S6-kinase

PD: Parkinson's disease

PI3-kinase: phosphatidylinositide 3-kinase

PLCδ1: phospholipase Cδ1

PTEN: phosphatase and tensin homolog deleted on chromosome ten

RA: retinoic acid

T101: 1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride

TGF-α: transforming growth factor alpha

TGF-β: transforming growth factor beta

THG: thapsigargin

tTG: tissue transglutaminase

VHL: von Hippel Lindau

Zeb1: zinc finger E-box binding homeobox 1

CHAPTER 1

Overview

Tissue transglutaminase (tTG) is a dual-function protein which couples an ability to bind and hydrolyze guanine triphosphate (GTP) with an enzymatic transamidation activity that allows it to generate covalent crosslinks between two protein substrates or between a protein and a polyamine (1-3). Tissue transglutaminase, which is also referred to as transglutaminase 2 (TGase-2 or TG2), is an 87 kilodalton protein encoded by the TGM2 gene located on human chromosome 20q11-12 (4). It is one of nine proteins belonging to the transglutaminase family whose other members include Factor XIII, Band 4.2, and TG1, TG3, TG4, TG5, TG6, and TG7. Each of these family members, with the exception of Band 4.2, exhibits enzymatic transamidation activity, but only tTG, TG3, and TG5 are known to bind GTP (2,5). Table 1.1 summarizes what is known about the tissue distribution, localization, biochemical activity and function of each of the various transglutaminase family members.

To date, most of the research carried out on the transglutaminases has been focused on Factor XIII, and TG1-3, while the remaining family members have not yet been fully characterized. Factor XIII circulates in plasma and is involved in fibrin stabilization and blood clotting (6). TG1 and TG3 are expressed primarily in keratinocytes and participate in the terminal differentiation of these cells (4). In contrast, the ubiquitous expression of tTG (TG2) has prompted studies of its function in many different cellular contexts. The results of these studies have shown that tTG participates in various physiological processes including differentiation, maintenance of the extracellular matrix, wound healing, cell migration, and

Table 1.1 Transglutaminase family members. Tissue transglutaminase (tTG or TG2) belongs to the transglutaminase family, whose other members include TG1, TG3-7, Factor XIII, and Band 4.2. The table highlights the tissue distribution, cellular localization, biochemical activities, and function of each family member. Note that some members are ubiquitously expressed, while others show specific tissue distributions. This table was adapted from that generated by Facchiano et al. (5).

Family Member	Tissue Distribution	Cellular Localization	Biochemical Activity	Function
TG1	Keratinocytes	Membrane	Transamidation	Differentiation
TG2 (tTG)	Ubiquitous	Cytosolic, ECM, Membrane, Nuclear	Transamidation GTP/GDP binding	Differentiation, Apoptosis, Cell signaling, ECM maintenance, Cell migration
TG3	Keratinocytes Hair Follicle	Cytosolic	Transamidation GTP/GDP binding	Differentiation
TG4	Prostate	Unknown	Transamidation	Reproduction
TG5	Ubiquitous	Membrane	Transamidation GTP/GDP binding	Differentiation
TG6	Unknown	Unknown	Transamidation	Not Characterized
TG7	Unknown	Unknown	Transamidation	Not Characterized
Factor XIII	Plasma	Extracellular	Transamidation	Blood coagulation, Wound healing
Band 4.2	Erythrocytes	Membrane	No Activity	Cytoskeletal network

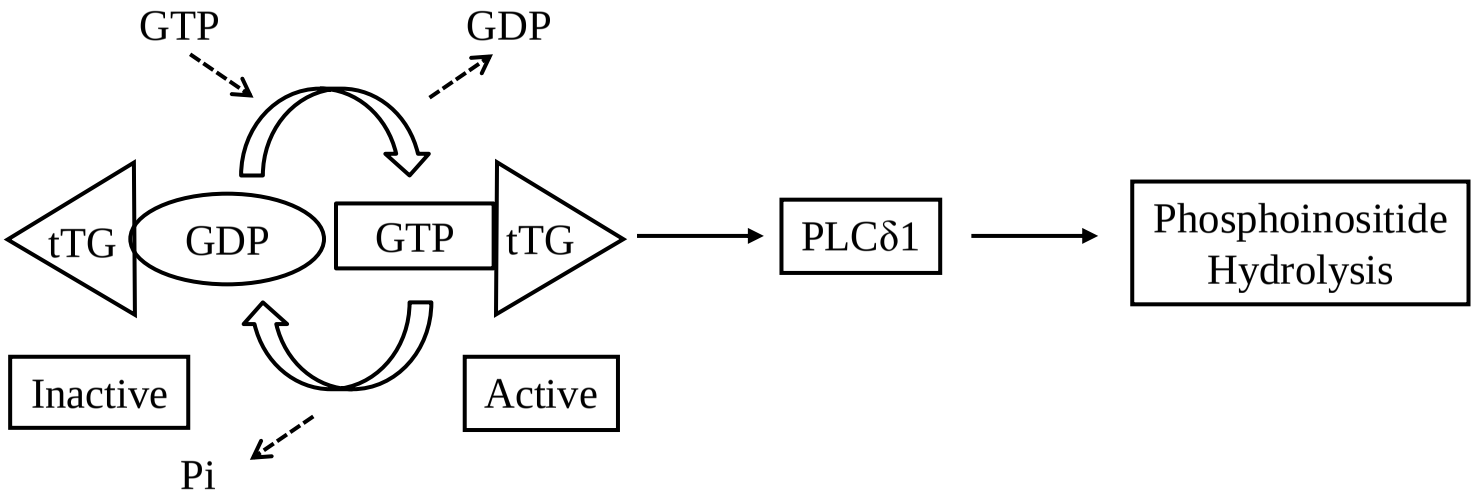
apoptosis (7-15). In addition, tTG expression and/or activation is often up-regulated in various human disease states including celiac disease, neurodegenerative disorders (Alzheimer's, Parkinson's, and Huntington's diseases), and cancer (16-22). A number of questions remain regarding how tTG is able to impact so many cellular processes and by extension, many disease states. What we have learned so far about the structure, function, and regulation of tTG has deepened our understanding of this protein and its importance to human disease. Moreover, it has given us reason to believe that this protein may be a worthy target for the development of therapies to combat these diseases.

Structure & Function

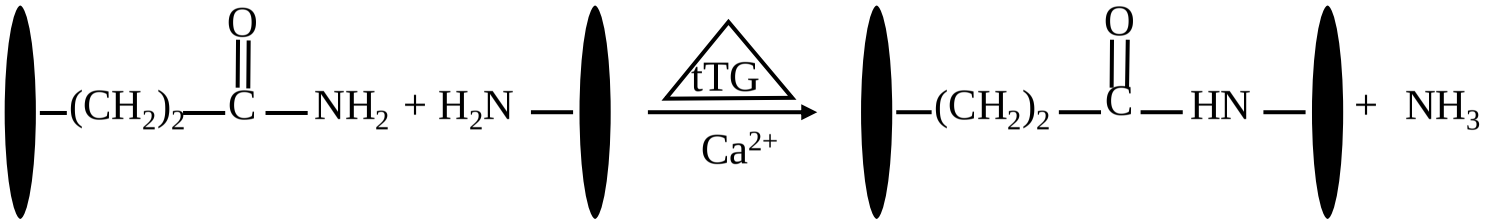
Our lab first became interested in tTG after it was identified in a proteomic screen looking for novel GTP-binding proteins (G-proteins) associated with the nucleus (23). While our interest in tTG was just beginning, other groups had been investigating tTG for some time and had classified it as a member of a new family of GTP binding proteins (called Gh) by showing that in rat liver tTG can bind and hydrolyze GTP and transduce signals from the α1B/D-adrenergic receptor to its downstream effector, phospholipase Cδ1 (3). A schematic of this activity is depicted in Figure 1.1, top panel. Interestingly, however, sequence analysis conducted on tTG revealed that it lacked the conventional guanine nucleotide-binding motif that is found in both families of large and small G-proteins, suggesting that tTG binds GTP via a unique mechanism. This idea was confirmed in 2002 when the first crystal structure of tTG bound to GDP was solved by our laboratory and it was discovered that tTG possesses a novel guanine nucleotide-interacting motif (24). While large G-proteins have a helical domain in the α subunit which promotes high affinity binding of guanine nucleotides, and small G-proteins instead use Mg^{2+} to facilitate nucleotide binding, the structure of tTG shows neither of these features

Figure 1.1 Enzymatic functions of tissue transglutaminase. tTG functions as both a GTPase and an acyl transferase which catalyzes the formation of protein crosslinks. The ability of tTG to bind and hydrolyze GTP serves to mediate signals from the α1B/D-adrenergic receptor to its downstream effector, phospholipase Cδ1 (PLCδ1), which results in phosphoinositide hydrolysis (top panel). In contrast, tTG's calcium-dependent transamidation activity leads to the generation of covalent crosslinks between a glutamine-containing protein substrate and a primary amino group found either in a lysine-containing protein substrate or in a polyamine (bottom panel).

GTP-binding



Transamidation



Protein with Glutamine Donor

Protein with Lysine Acceptor

Cross-linked Proteins

(25,26). Instead, a series of positively-charged residues surround the nucleotide-binding region, the most important of which is Arg580, because it forms hydrogen bonds with the guanine ring and the α- and β-phosphates of GDP.

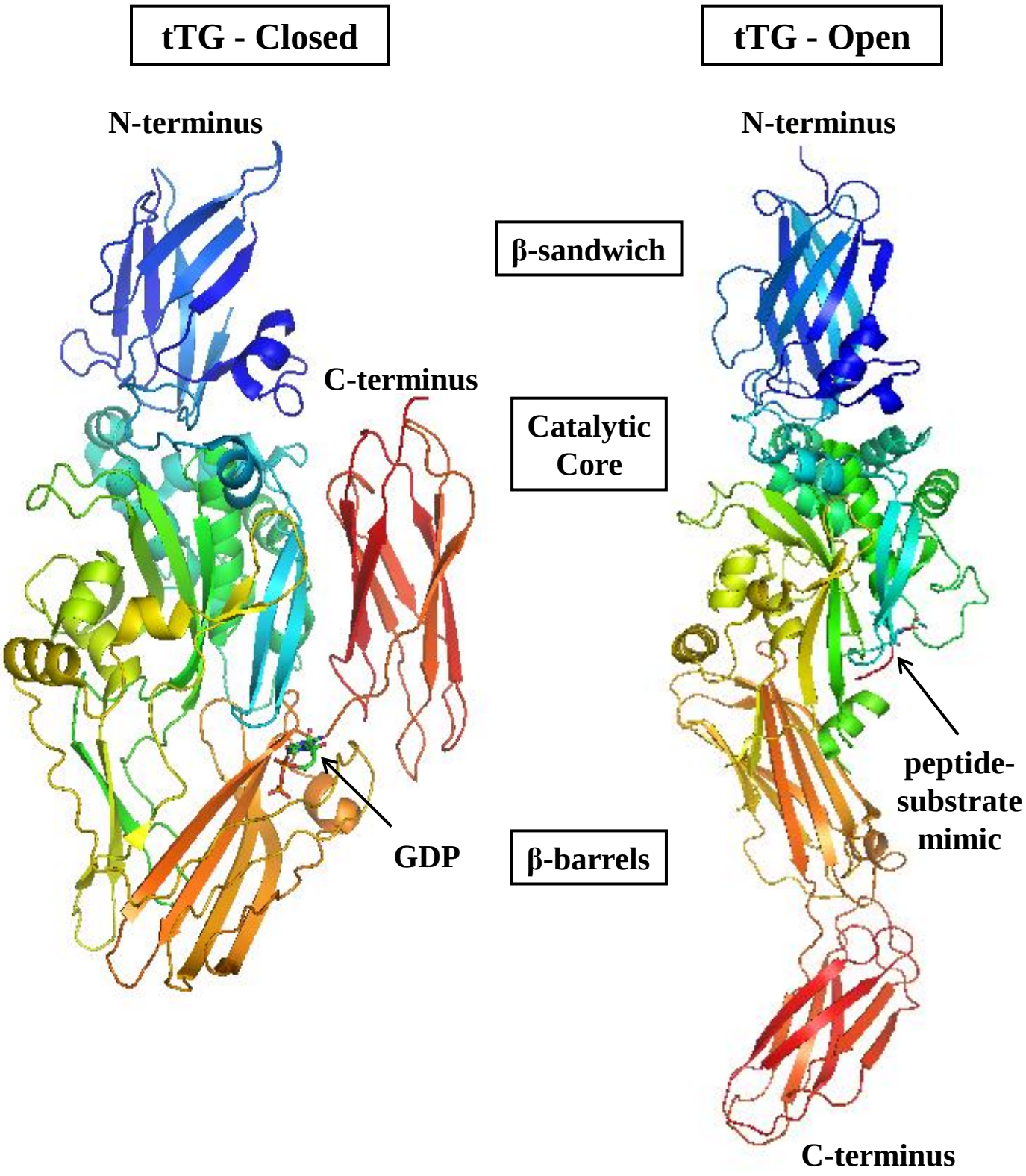
In contrast to the extensively studied GTPase function of the more conventional large and small families of G-proteins, very little is known about the regulation of tTG's GTPase activity and how this function contributes to tTG's role in cellular processes. Researchers have yet to determine whether there is a specific guanine nucleotide exchange factor (GEF) or GTPase activating protein (GAP) for tTG or whether such regulation is even needed for tTG to bind and hydrolyze GTP.

Instead, most of the research conducted on tTG to date has focused on its function as an acyl transferase. In a calcium-dependent transamidation reaction, tTG catalyzes the formation of amide bonds (crosslinks) between the carboximide group of a protein-bound glutamine and a primary amino group, which can be found in the ε-amino group of a protein-bound lysine residue or in a polyamine (Figure 1.1, bottom panel) (1,2). Studies have revealed that tTG, as well as nearly all of its closely related family members (see Table 1.1), shares a catalytic triad motif with members of the cysteine protease family, consisting of residues Cys277, His335, and Asp358. The reaction takes place in two steps, with the first being the transient acylation of Cys277 when it performs a nucleophilic attack on the glutamine-containing substrate and generates an acyl-thiolate intermediate. The second step involves the primary amino group of the second substrate executing a nucleophilic attack resulting in the deacylation of Cys277 and the release of the crosslinked products and one molecule of ammonia. Mutation of Cys277 results in a form of tTG that is unable to carry-out this crosslinking reaction, thus confirming that this residue is critical for the transamidation activity (27,28). Interestingly, Band 4.2, the only transglutaminase

family member without crosslinking activity (see Table 1.1) has an alanine instead of a cysteine at position 277, further demonstrating the importance of this residue in catalyzing the transamidation reaction. Likewise, mutation of His335 or Asp358, the other residues that make up the catalytic triad, also makes tTG transamidation-defective (1). The crystal structure of tTG solved in 2007 showing it bound to an inhibitor Ac-P(DON)LPF-NH2 (where DON is the electrophilic amino acid 6-diazo-5-oxo-L-norleucine) which mimics a natural PQLPY motif found in gluten peptides, further supports this mechanism and the involvement of this catalytic triad (29). Much of the work performed on tTG up to this point has been focused on this activity of tTG, in part because of access to crosslinking inhibitors, such as monodansylcadaverine (MDC) and 1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride (T101).

In addition to confirming mechanistic details regarding tTG's biochemical activities, the crystal structures of tTG revealed that it can adopt two drastically different conformations; an open conformation and a closed conformation (Figure 1.2). tTG possesses four structural domains: an N-terminal β-sandwich domain, the catalytic core which harbors the nucleotide binding site and the transamidation active site, followed by two C-terminal β-barrel domains (30). In Figure 1.2, the structure on the left, which was solved by our laboratory, shows tTG bound to GDP (24). The two C-terminal β-barrels are folded over the catalytic core, thus preventing transamidation substrates from accessing the active site. For this reason, this conformation has been termed the "closed" conformation of tTG. In contrast, the crystal structure of tTG on the right, solved by Pinkas et al., shows it bound to an inhibitor which mimics its gluten peptide substrate, and it adopts an essentially linear conformation where the two C-terminal β-barrels of tTG have swung away from the catalytic core to provide complete

Figure 1.2 Crystal structures of tTG. Two crystal structures of tTG have been solved and have shown that tTG can adopt drastically different conformations. The structure on the left depicts the "closed" conformation of tTG which occurs when tTG is bound to GTP or GDP (in this case, GDP). In this structure, the C-terminal β-barrels fold over the catalytic core and block access to the transamidation active site. The structure on the right, represents the "open" conformation of tTG, with tTG bound to a peptide-substrate mimic. When tTG reaches a nucleotide-free state, the C-terminal β-barrels swing away from the catalytic core and provide substrates with access to the transamidation active site.


tTG - Closed
tTG - Open
N-terminus
N-terminus
β-sandwich
C-terminus
Catalytic Core
peptide-substrate mimic
GDP
β-barrels
C-terminus

access of substrates to the active site (where the inhibitor/substrate was shown to bind) (29). Therefore, this conformation of tTG has been called the "open" conformation.

Solving the open and closed structures of tTG helped solidify the hypothesis that the GTP-binding and enzymatic transamidation activities of tTG reciprocally regulate one another, such that when tTG is bound to nucleotide, its transamidation function is inactive. However, when tTG achieves a nucleotide-free state, and is able to bind calcium, it can function as an acyl transferase. This was supported by studies using rat tTG, whereby it was shown that the binding of guanine nucleotides (either GTP or GDP) to tTG inhibited its transamidation activity (3). Additionally, research analyzing the binding of GDP or GTPγS (a non-hydrolyzable form of GTP) to recombinant human tTG revealed a dose-dependent inhibition of tTG's transamidation activity (31). This reciprocal regulation of tTG's enzymatic functions, as well as the detailed information provided by solving the crystal structures that show the open and closed conformations of tTG, presented our laboratory and others with a unique opportunity, namely, to mutate specific residues that are important for tTG to carry-out a specific biochemical activity (i.e. to bind GTP or GDP or crosslink) and determine how these changes would impact cellular behavior.

With this idea in mind, our laboratory mutated wild-type tTG, which has a Kd of 400 nM for binding to GTP, to generate a form of tTG that was no longer capable of binding guanine nucleotides. Specifically, Arg580, a residue which makes critical contacts with GDP, was changed to a lysine (tTG R580K) (32). Although incapable of binding nucleotide, this mutant retained its transamidation activity. Surprisingly, when ectopically expressed in a variety of cell types, this mutant induced cell death, unlike wild-type tTG which cells tolerate well. With the idea that this apoptotic response was due to the fact that the tTG R580K mutant exhibited high,

un-regulated transamidation activity, a double mutant was made where both Arg580 and Cys277 were changed. Cys277 is the critical cysteine residue in the transamidation active site (the catalytic triad), that when mutated, completely eliminates transamidation activity (27). However, comparing the effects of ectopically expressing the single mutant (tTG R580K) or the double mutant (tTG R580K/C277A) in cells revealed that both mutant forms of tTG induced a comparable amount of cell death (32). Thus, the apoptotic response induced by ectopically expressing the nucleotide-binding defective form of tTG in cells is not the result of aberrant crosslinking activity. These findings have led us to propose a new hypothesis as to why the mutant form of tTG (tTG R580K) induces cell death. Given that this mutant cannot bind nucleotide, we believe that it adopts a more open conformation and that this structure is harmful to cells. When tTG opens up, it might bind to a protein(s) necessary for cell viability, perhaps inactivating it or preventing it from localizing properly, thus compromising cell survival. Work is ongoing in the laboratory to test these hypotheses and better understand how these mutations impact tTG function in cells and contribute to the observed changes in cellular outcomes.

The role of tissue transglutaminase in apoptosis

Ten years ago, the perception of tTG's role in biology was very different than what it is today. tTG was originally studied for its role in promoting apoptosis, as researchers observed that increased tTG expression was correlated with cells undergoing cell death (33-36). One of the first examples of this was found using models of induced liver hyperplasia, which mimic the natural cycle of proliferation and cell death that occurs when the liver is exposed to an acute injury (37). In this model, rat livers are injected with a dose of lead nitrate which triggers the proliferation of hepatocytes to compensate for the injured cells. After 3-4 days, the level of lead nitrate diminishes and programmed cell death occurs to restore the liver to its normal mass.

Examination of tTG in this model revealed that its expression and activation were up-regulated, specifically during the programmed cell death stage, and that it was an important contributor to the apoptotic response (38). In other studies, this correlation between increases in tTG expression and the induction of apoptosis was thought to be the result of enhanced tTG crosslinking activity resulting in protein aggregation, or in some cases, impaired mitochondrial function or enhanced oxidative stress (35,39).

Around the same time that tTG was being linked to the apoptotic response during liver hyperplasia, researchers were also finding a unique connection between tTG and certain neurodegenerative diseases including Alzheimer's (AD), Parkinson's (PD), and Huntington's disease (HD). These disorders are characterized by the accumulation of protein aggregates which promote neuronal dysfunction and death (40). In each of these diseases a specific protein(s) is mutated in such a way as to promote its aggregation including β-amyloid and Tau in AD, α-synuclein in PD, and the Huntington protein in HD. Surprisingly, all of these proteins were identified as potential substrates of tTG *in vitro* and tTG was shown to be a component of the aggregates that form in the brains of patients afflicted with these disease states (19-21,41,42). Perhaps the best studied examples involve those proteins that undergo poly-glutamine expansions, such as the Huntington protein. Given tTG's ability to crosslink glutamine-containing proteins, these seemed to be ideal candidates for tTG's transamidation activity. Normal individuals have between 6 and 39 glutamine (CAG) repeats within the amino terminus of the Huntington protein, whereas individuals with Huntington's disease have more than 39 CAG repeats in the Huntington protein, with some of the most severe cases reaching glutamine expansions of more than 60 residues (43). Researchers showed that the levels of tTG expression were elevated in cells expressing a mutant form of the Huntington protein and that tTG

preferentially crosslinks the poly-glutamine-expanded form of Huntington (a protein with more than 39 glutamine residues) to a much greater extent than the wild-type protein (a protein with 6-39 glutamine residues), thus causing significant protein aggregation (41,44). Based on these findings, many researchers became interested in tTG and how its activity toward these particular substrates could impact neurodegenerative disorders.

In addition to these proteins serving as possible substrates for tTG, its expression and/or transamidation activity were shown to be up-regulated in the brains of individuals affected by these neurodegenerative diseases (19,20,41). Researchers discovered that inhibition of tTG's crosslinking activity reduced protein aggregation and extended the lifespan of affected mice in models of PD and HD (45-47). Cumulatively, these studies portrayed a role for tTG in crosslinking specific protein substrates to drive protein aggregation and cell death. Moreover, they laid the foundation for future studies to determine whether tTG may represent a valid target for therapeutic intervention against neurodegenerative diseases characterized by protein aggregation.

The role of tissue transglutaminase in cellular differentiation

While much of the early work on tTG suggested that it was important for triggering cell death, other laboratories including our own, became interested in the idea that tTG may also be important in promoting cellular differentiation. Several studies have shown that tTG expression is up-regulated during cellular differentiation (9,14,48). For example, treatment of SH-SY5Y neuroblastoma cells with retinoic acid (RA), a natural compound known to induce the differentiation of these cells into neurons, resulted in increased expression of tTG and its associated transamidation activity (48). Two pieces of evidence suggested that this increase in tTG expression and activation were not just casual markers of cellular differentiation, but instead

played critical roles in mediating this outcome. First, it was shown that inhibiting tTG transamidation activity, by stably expressing a transamidation-defective mutant of tTG (tTG C277S) in cells or by knocking-down tTG expression using an anti-sense construct, prevented RA from inducing differentiation. Additionally, ectopic expression of tTG in SH-SY5Y cells was sufficient to mimic the effects of RA and induce neurite outgrowth, an indicator of cellular differentiation. These results suggested that the up-regulation in tTG expression and transamidation activity was responsible for RA's ability to promote cellular differentiation, highlighting the possibility that tTG may play an important role in promoting cellular processes besides apoptosis.

It was about this same time that our laboratory began evaluating the impact of natural and synthetic retinoids on cancer progression, as there was much interest in the use of these agents as potential cancer therapies due to their ability to cause cells to differentiate. Using human HL60 leukemia cells as a model system, we showed that RA treatment caused the cells to differentiate and inhibited their growth, whereas treatment of the cells with a synthetic analog of RA known as N-(4-hydroxyphenol)retinamide (HPR) potently induced cell death (14). The only difference between these two retinoids is the addition of a phenol ring to the carbon backbone of RA. An interesting clue as to how these retinoids gave rise to different cellular outcomes (cell differentiation versus cell death) came from the finding that long-term (2 days) pre-treatment of HL60 cells with RA was sufficient to protect the cells from HPR-induced apoptosis (14). This raised the possibility that RA treatment likely caused changes in the expression of certain genes which could protect cells against HPR-mediated apoptosis. Indeed, this turned out to be the case, as tTG was identified as the gene specifically up-regulated by RA to protect cells from HPR-induced cell death. Moreover, ectopic expression of tTG in HL60 cells was as efficient as RA

treatment at protecting cells from HPR-induced apoptosis, and blocking tTG activity with MDC, converted RA from an inducer of cellular differentiation into a cell death factor. Thus, these studies not only linked tTG expression and activation to the induction of cellular differentiation, but also showed for the first time that in certain cellular contexts tTG up-regulation can help protect cells against apoptotic challenges.

The role of tissue transglutaminase in cell survival

These two opposing roles for tTG, one in promoting apoptosis in some cellular contexts, while protecting cells undergoing differentiation from cell death in others, was emerging while other groups were starting to explore the influence of tTG in human cancer progression. Unexpectedly, tTG expression and/or its transamidation activity were found to be up-regulated in a number of different types of human cancer including breast, brain, pancreatic, and ovarian cancer (17,49-52). In particular, the expression and activation of tTG is most elevated in those cancers that are highly aggressive, metastatic, and chemo-resistant (17,50,53-55). Thus, given the protective role that tTG plays during cellular differentiation, the obvious question that arose was whether tTG would have a similar protective role in a cancer cell.

Indeed, it was shown that tTG could contribute to the survival of human cancer cells challenged with various stress conditions. For example, MDA-MB231 cells, an aggressive human breast cancer cell line, were shown to express inordinately high levels of tTG and exhibited constitutive transamidation activity. These cells grew at a rapid rate and were known to be resistant to several traditional chemotherapeutic agents, including doxorubicin. However, upon knocking-down tTG expression using siRNAs or inhibiting its crosslinking activity using the tTG-specific inhibitor, monodansylcadaverine (MDC), these cells became sensitized to treatment with doxorubicin and showed enhanced apoptosis (53,56).

An analogous study conducted in ovarian cancer cells investigated the role of tTG in the development of resistance to another chemotherapeutic agent, cisplatin. Researchers had previously found that tTG expression and activity was up-regulated in over 70% of ovarian tumor samples that were analyzed (50). Based on this, they then went on to show that those ovarian cancer cell lines with the highest level of tTG expression were also the most resistant to cisplatin (54). Knocking-down tTG expression using an anti-sense construct or inhibiting its transamidation activity with a recently generated inhibitor, KCC009, sensitized the cells to cisplatin, thus confirming what had been shown earlier in MDA-MB231 cells, namely that tTG protects cells from apoptotic challenges and may be important in the development of chemo-resistance.

In addition, tTG was shown to play an important role in promoting the epidermal growth factor (EGF)-stimulated growth and survival of cells. The EGF receptor is a well-studied cell surface receptor tyrosine kinase, whose signaling capabilities have been intimately linked to oncogenesis (57,58). The EGF receptor is over-expressed in a number of different types of human cancer, and upon binding ligand (EGF) it becomes activated and sends aberrant signals that trigger cancer progression. Thus, it is not surprising that the EGFR has been major target for therapeutic intervention for many years. The interplay between EGF receptor signaling and tTG was first discovered in human SKBR3 breast cancer cells, where tTG expression was increased in response to EGF treatment from nearly undetectable levels to levels that were readily detectable by Western blot analysis (53). These cells also grew and survived serum-starvation-induced apoptosis better when treated with EGF. Importantly, these effects were shown to be dependent on tTG, as inhibition of tTG's crosslinking activity using MDC prevented the growth advantage and protective effect afforded to SKBR3 cells by EGF stimulation. Likewise, EGF

stimulation of two other human breast cancer cell lines, BT-20 and MDA-MB468 cells, caused an up-regulation of tTG expression suggesting that the up-regulation and activation of tTG may be a fundamental mechanism by which EGF protects cells, especially cancer cells, against apoptotic challenges (27,53).

How is tTG able to impact cell viability? Given the evidence that tTG's transamidation activity was important for promoting cell survival, it was logical to suspect that tTG must be crosslinking a particular substrate to impact cell viability. While many efforts have been made to identify such proteins, determining those that are physiologically relevant has proven to be a challenging task. However, two protein substrates of tTG have been discovered in recent years which may have a critical impact on cancer cell survival. One such substrate is caspase-3, a cysteine protease that is part of the conventional apoptotic signaling cascade (59). In an actively growing cell, caspase-3 exists as a pro-caspase, or inactive enzyme. However, if a cell should decide to undergo programmed cell death (i.e. in the face of an extreme cellular insult that has damaged the cell beyond repair), caspase-3 becomes cleaved to yield an active cysteine protease which then cleaves several essential proteins necessary for cell survival, thus resulting in cell death. The finding that tTG could crosslink caspase-3 came from a study that was investigating how Bax-deficient HCT-116 colon cancer cells were able to survive amidst treatment with thapsigargin (THG), an inhibitor of the endoplasmic reticulum calcium ATPase (60). The study found that THG treatment resulted in the formation of higher molecular weight forms of pro-caspase-3. Knocking-down tTG using siRNAs or inhibiting its transamidation activity using MDC not only blocked the THG-induced formation of these higher molecular weight species of pro-caspase-3, but it also sensitized the cells to apoptosis. These results indicate that THG

treatment of HCT-116 cancer cells results in the up-regulation and activation of tTG, which in turn protects cells from THG-triggered apoptosis by crosslinking and inactivating caspase-3.

In addition to caspase-3, tTG was also found to crosslink IκBα, the major negative regulator of NF-κB (61). NF-κB is a well-studied transcription factor which is sequestered in the cytosol through its association with its regulatory subunit IκBα (62). However, upon growth factor treatment or in response to cellular stresses, IκBα is phosphorylated by the IκB kinase (IKK) complex, an event which targets it for ubiquitination and subsequent degradation in the proteasome. This, in turn, frees NF-κB to translocate to the nucleus where it enhances the transcription of various genes involved in promoting cell growth and survival. In recent years, NF-κB has become recognized as an important contributor to tumorigenesis, as its aberrant activation has been detected in a number of different cancer types (62-66). One mechanism by which NF-κB is activated in cancer cells involves tTG and its ability to crosslink IκBα (61). The observation that increases in tTG expression correlated with high NF-κB activity was made in several different cancer cell lines including, MDA-MB231 breast cancer cells, A375 malignant melanoma cells, and Panc-28 pancreatic cancer cells. It was then shown that knocking-down tTG or inhibiting its transamidation activity in each of these cell lines resulted in a significant reduction in NF-κB activity, implying that NF-κB activation was functionally dependent on tTG. The authors went on to show that tTG could crosslink IκBα into higher molecular weight species of the protein both *in vivo* and *in vitro*. Moreover, recombinant IκBα crosslinked by tTG displayed weaker binding to NF-κB than non-crosslinked IκBα, implying that tTG works to inactivate the regulatory role of IκBα by crosslinking it, rendering NF-κB consitutively active.

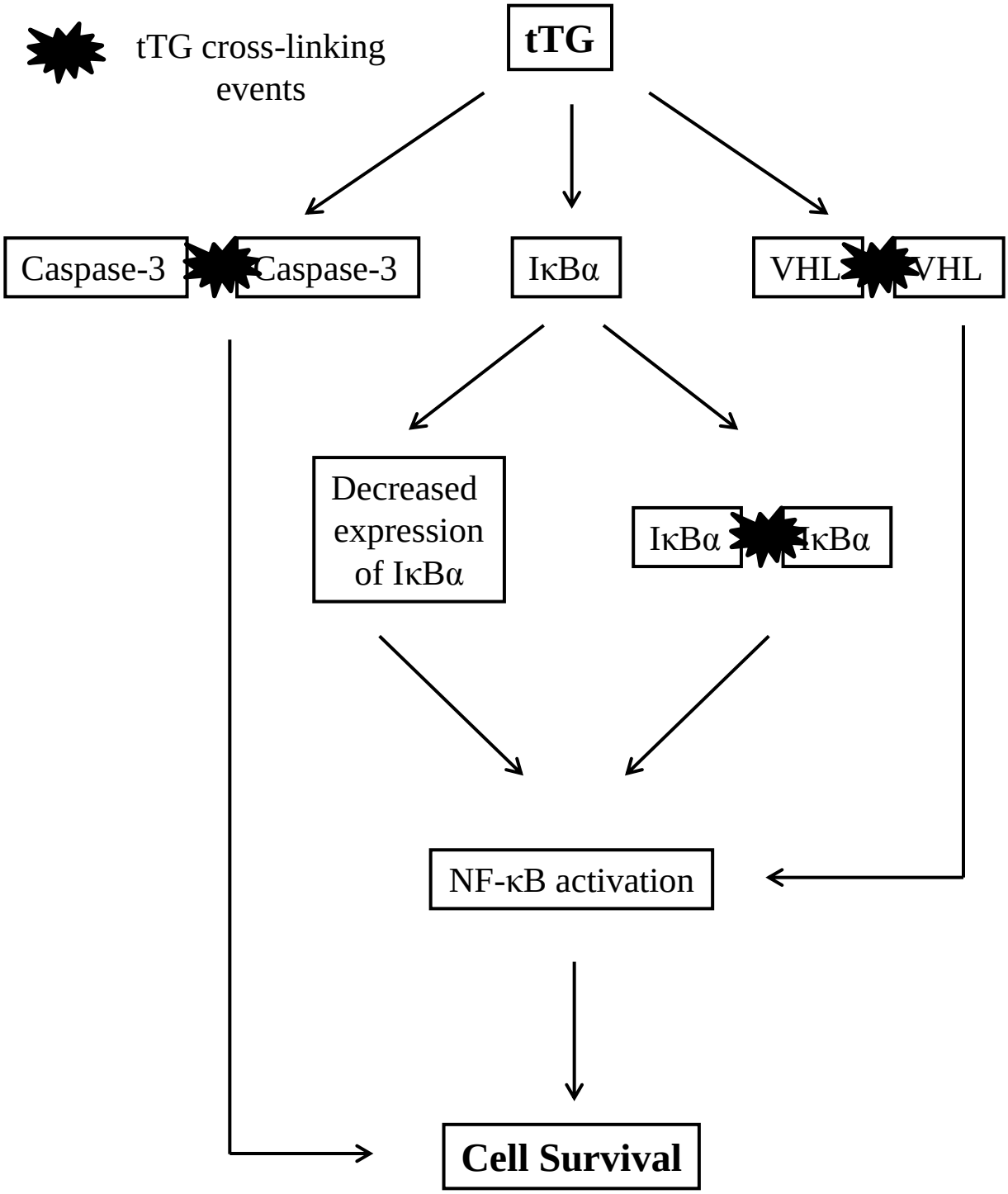
This connection between tTG and NF-κB activation has been observed by other groups in ovarian and breast cancer cells as well (54,67). However, the mechanism through which tTG

activates NF-κB does not always appear to involve the crosslinking of IκBα into a non-functional oligomer. Rather, in some cases, increases in tTG expression appear to lead to decreases in the expression of IκBα, while knocking-down tTG using siRNAs or inhibiting its crosslinking activity enhances expression of IκBα, which then down-regulates NF-κB activity (54). How tTG regulates the expression of IκBα is currently unknown, but recent evidence suggesting that tTG can localize to the nucleus and influence the expression of metabolic enzymes, opens the door to the possibility that tTG may directly influence IκBα expression (68,69). However, at least one other study has described another potential substrate for tTG, the tumor suppressor von Hippel-Lindau (VHL), which, when post-translationally modified by tTG, may lead to NF-κB activation (67). VHL is part of an E3 ubiquitin ligase complex which targets hypoxia inducible factor 1 alpha (HIF1α) for degradation by the proteasome (70,71). Loss of VHL results in the accumulation of HIF1α and increased signaling through transforming growth factor alpha (TGFα) and EGF, resulting in NF-κB activation. At least one group has shown that tTG drives the formation of VHL polymers which are subsequently targeted for degradation by the proteosome (67). Thus, tTG's ability to limit the expression of VHL would allow for enhanced NF-κB signaling. Overall, these findings depicted in Figure 1.3, show that tTG is commonly up-regulated and activated in cancer cells to promote cell viability and may achieve this through multiple mechanisms such as inactivating components of the apoptotic machinery, or activating keys nodes of various signaling networks.

The role of tissue transglutaminase in aberrant cellular growth

In addition to its role in protecting cells from apoptotic challenges, there is evidence to suggest that tTG may also contribute to malignant transformation by promoting several other cellular behaviors commonly exhibited by cancer cells. One such example was discovered in our

Figure 1.3 tTG promotes cell survival through various mechanisms. One of tTG's primary roles in promoting cancer progression is to enhance cell survival. tTG is able to crosslink Caspase-3 and inactivate it, thus preventing cells from undergoing programmed cell death. In other cellular contexts, tTG has been shown to regulate IκBα. tTG has been shown to directly influence IκBα expression levels through a poorly understood mechanism. However, tTG can also crosslink IκBα into higher order oligomers which prevents it from binding to NF-κB and inhibiting the ability of NF-κB to function as a transcription factor. Both of these mechanisms lead to the constitutive activation of NF-κB. Similarly, tTG has been shown to crosslink the tumor suppressor, von Hippel Lindau (VHL) and inactivate it, leading to NF-κB activation. All of these are mechanisms by which tTG enhances cell survival.


tTG cross-linking events
tTG
Caspase-3
Caspase-3
IκBα
VHL
VHL
Decreased expression of IκBα
IκBα
IκBα
NF-κB activation
Cell Survival

laboratory using the human breast cancer cell line, SKBR3. As mentioned previously, EGF treatment induces tTG expression and activation in these cells, as well as other breast cancer cells, including BT-20 and MDA-MB468 cells, and this up-regulation in tTG expression/activation is critical for promoting chemo-resistance (53). To learn more about how EGF treatment causes an up-regulation of tTG expression, our laboratory transiently transfected SKBR3 cells with plasmids encoding constitutively active (or fast cycling) mutant forms of small GTPases which are known to be downstream effectors of the EGF receptor including Ras (G12V), RhoA (F30L), and Cdc42 (F28L) (27). Interestingly, while introduction of any one of these activated small GTPases alone in the cells was insufficient to cause an up-regulation of tTG expression, combinations of these plasmids, such as Ras (G12V) with either RhoA (F30L) or Cdc42 (F28L), resulted in a marked increase in tTG expression. Thus, these results suggested that the induction of tTG expression by EGF requires inputs from multiple signaling pathways such as the Ras and Rho/Cdc42 pathways. Importantly, these findings also correlate with the observation that the highest levels of protein expression of tTG often occur in the most aggressive, metastatic, and invasive tumors (i.e. those that have acquired many mutations in signaling proteins and likely display persistent activation of multiple signaling pathways) (17,50,53-55).

With a preliminary understanding of the signaling events that are responsible for increasing tTG expression levels in cancer cells, the next goal was to determine how tTG expression impacts the behavior of the cells. We found that ectopic expression of tTG in these cells is sufficient to mimic the effects of EGF and leads to enhanced anchorage-independent growth, as read-out by soft agar colony formation (an *in vitro* measure of tumorigenicity) (27). Interestingly, ectopic expression of a transamidation-defective form of tTG in which the active

site cysteine has been mutated to a valine (C277V) was unable to induce anchorage-independent growth like wild-type tTG. Examination of the activities of various signaling proteins in cells over-expressing tTG led to the discovery that tTG stimulates the activation of the non-receptor tyrosine kinase c-Src. Moreover, inhibition of Src activity using the inhibitor, PP2, eliminated tTG-promoted soft agar growth, thus confirming this as the mechanism by which tTG transforms these cells. Further investigation revealed that wild-type tTG, but not a transamidation-defective form of tTG (tTG C277V), binds Src and the intermediate filament, keratin 19 in SKBR3 cells, resulting in the formation of a ternary complex which is likely responsible for relieving the auto-inhibition of Src and allowing it to open and become an active kinase. This was the first clue that tTG may potentially act as a scaffold to regulate signaling proteins within the cell that are important for cellular transformation.

In addition, our laboratory discovered a unique way in which tTG can help drive progression of the malignant state, namely through its ability to be released from cells in novel cellular structures called microvesicles (72). Microvesicles are small vesicle compartments that arise independently of the classical secretion pathway (i.e. ER-Golgi trafficking) and appear to bud directly from the plasma membranes of various cancer cells (73,74). They are believed to represent a novel form of cell-cell communication, as they can be released from one cell and taken up by neighboring cells. Their contents include some very interesting and important cargo including cell surface receptors (i.e. the EGF receptor), metabolic proteins, molecular chaperones, RNA transcripts, and microRNAs (72-76). In the case of cancer cells shedding microvesicles, it is thought that the transfer of this cargo is important for re-programming recipient cells in ways that help drive the oncogenic state. For example, if the recipient cell happens to be another cancer cell, the microvesicles can stimulate their growth and survival

(75,76). Moreover, microvesicles have also been found in the circulation of cancer patients where they have been recently suggested to play a role in the establishment of the pre-metastatic niche (75-77). Thus, microvesicles can contribute to primary tumor growth, as well as promote metastasis.

Our laboratory showed that certain cancer cell lines, such as HeLa cervical carcinoma cells, require EGF treatment to induce the formation of microvesicles, whereas other more highly aggressive cancer cells such as MDA-MB231 breast cancer cells and U87 glioblastoma cells constitutively generate microvesicles (72). When these microvesicles were collected and added to normal (non-transformed) NIH3T3 fibroblasts, the cells started to behave like cancer cells, as evidenced by their acquired ability to form colonies in soft agar compared to the control NIH3T3 fibroblasts not treated with microvesicles. Proteomic analysis of the contents of these microvesicles from MDA-MB231 cells and U87 cells led to the discovery that not only was tTG a major protein component, but that its primary extracellular binding partner, fibronectin, was one of the most abundant proteins in the microvesicles. This prompted our laboratory to look at whether tTG and fibronectin could be working together to mediate the transforming activity of these microvesicles. Indeed, it was shown that in the microvesicles, tTG crosslinked fibronectin and that the interaction of the microvesicle-associated, cross-linked fibronectin with recipient cells resulted in enhanced integrin signaling and the induction of cellular transformation. Treatment of these microvesicles with inhibitors against tTG crosslinking activity or with an RGD peptide which blocks the ability of fibronectin to bind to integrins, eliminated the transforming potential of the microvesicles. Thus, these results demonstrate a unique way in which tTG influences cellular transformation, namely through its role in enabling microvesicles to dock onto neighboring cells to enhance their integrin-coupled signaling activities.

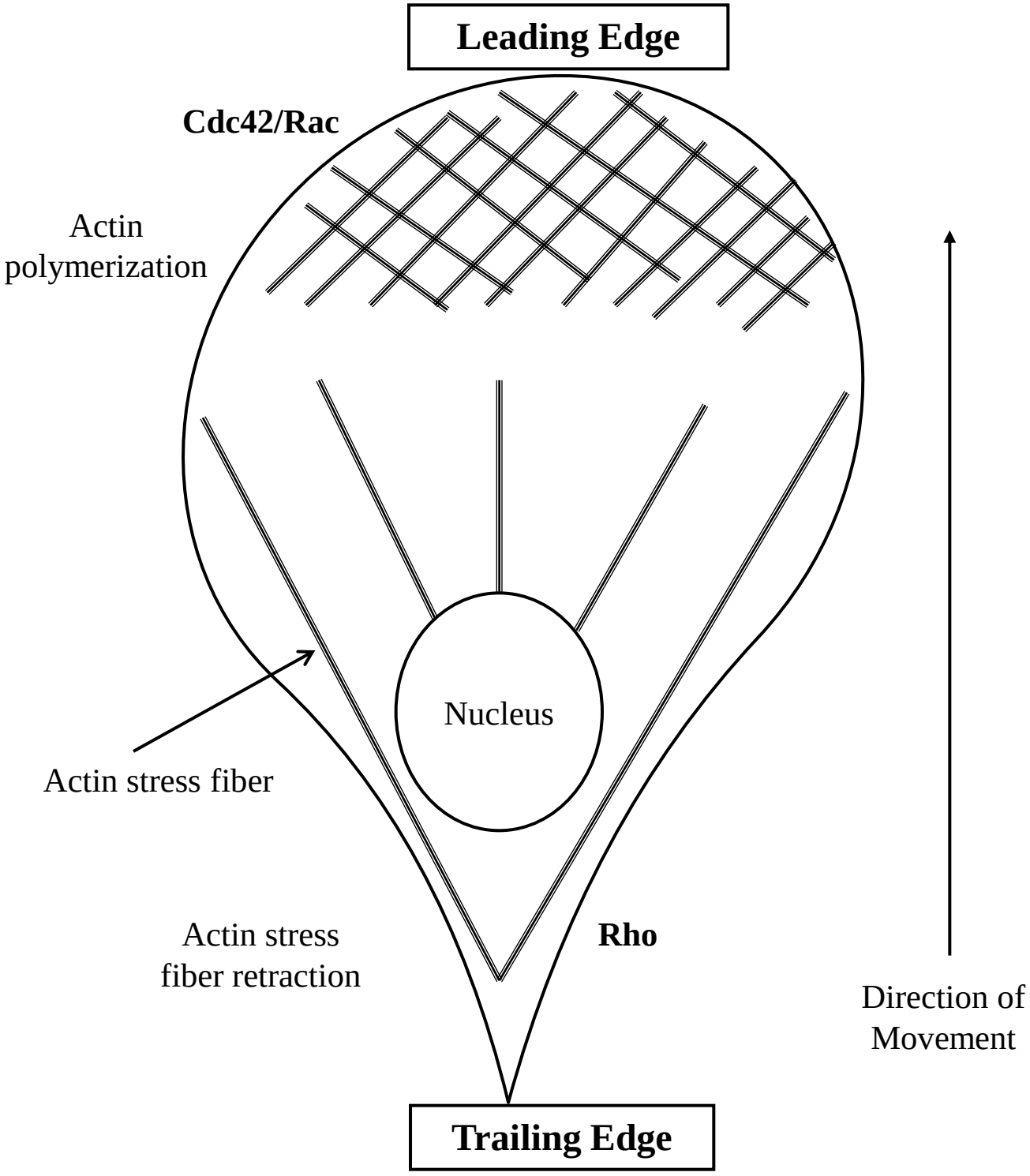
The role of tissue transglutaminase in cell migration

The ability of cells to migrate is an important feature for many aspects of biology including embryonic development, tissue repair, and immune responses (78-82). Moreover, it is a cellular process often studied in human cancer because its de-regulation is important for allowing aggressive cancer cells to acquire enhanced invasive and metastatic potential (83,84). Cell migration is regulated through the coordination of signaling and cytoskeletal proteins which work together to shape the cell and direct its movement. For directional cell migration to occur, a cell must undergo polarization to establish a front or leading edge of the cell and a back or trailing edge (85), as diagrammed in Figure 1.4. At the leading edge, actin polymerization results in membrane protrusions which extend the cell in the direction of movement. New contacts are made between these protrusions and the underlying substrate, thus giving the cell an adhesive capability for movement. However, in order for the cell to move, these changes at the leading edge must be coupled with contraction of the actomyosin cytoskeleton and severing of the previously established adhesions. The modifications at the trailing edge will then allow the cell to detach from its substrate, retract, and pull the cell body toward the leading edge.

Many proteins have been shown to play a role in cell migration, including the classic small GTPases, Cdc42, Rac, and Rho (86,87). It has been well-established that Cdc42 plays a key role in maintaining cell polarity and helps to recruit membrane/signaling proteins to the leading edge to propel the cell forward. Rac also localizes to the leading edge where it promotes actin polymerization; whereas Rho can be found at the rear of the cell where it functions to facilitate retraction.

Cell migration is a normal process that cells undergo during development or other times to maintain tissue homeostasis. However, aberrant cell migration is a key contributing factor to

Figure 1.4 Structure of a migrating cancer cell. In order for directional cell migration to occur, a cell must become polarized in such a way as to establish a front or leading edge of the cell and a back or trailing edge of the cell. Actin polymerization at the leading edge is responsible for extending the cell in direction of movement, whereas actin stress fiber formation facilitates retraction at the trailing edge to allow the cell to detach and move forward. Rac localizes to the leading edge to facilitate actin polymerization. Cdc42 localizes to the cytoplasm and leading edge and plays a role in maintaining cell polarity during migration. Rho can be found at the trailing edge where it promotes retraction of the actin stress fiber network.


Leading Edge
Cdc42/Rac
Actin
polymerization
Nucleus
Actin stress fiber
Actin stress
fiber retraction
Rho
Direction of
Movement
Trailing Edge

the metastatic and invasive potential of human cancer cells. For cancer cells to metastasize to other tissues, several steps must take place (88). First, cells must migrate from the site of origin and acquire the ability to invade through the tissue. They undergo intravasation and enter into the vasculature, followed by circulation through the bloodstream or the lymphatic system. The cells then complete extravasation where they exit the vasculature and colonize a secondary site. Most of the research regarding this process has been devoted to understanding the first step, namely how cells acquire the ability to migrate and invade.

One of the first studies linking tTG to cell migration was in 2006 when researchers were generating MDA-MB231-derived sub-lines on the basis of their differential expression of tTG (56). They discovered that the MDA-MB231 sub-line expressing high levels of tTG showed enhanced cell attachment and spreading on fibronectin-coated surfaces, compared to another sub-line which did not express tTG. In addition, tTG was shown to localize along the plasma membranes of these cells where it associated with β1, β4, and β5 integrins. Integrins are trans-membrane receptors which coordinate interactions between the extracellular matrix and the actin cytoskeleton during cell migration (89,90). Upon ligand binding, integrins transduce signals from the cell surface to adhesion complexes resulting in the activation of the Rho family of GTPases which regulate actin dynamics to promote cell motility. Other studies confirmed these initial findings by demonstrating that tTG can bind to integrins to mediate the interaction between integrins and fibronectin and enhance cell adhesion and migration (50,91,92). The ability of tTG to interact with integrins and serve as a co-receptor for fibronectin did not require its crosslinking activity, given that there was no difference between wild-type tTG and the transamidation-defective form of tTG (tTG C277S) in assays of cell adhesion and migration (92). However, other groups went on to show that tTG's transamidation activity could be important

for integrin signaling in hepatocytes and osteoblasts, as tTG can crosslink fibronectin into higher molecular weight polymers (93,94). They demonstrated that the crosslinked form of fibronectin enhanced integrin signaling and led to increased cell attachment.

Our laboratory demonstrated the importance of tTG in cell migration when analyzing EGF-receptor signaling in HeLa cervical carcinoma cells (13). Antonyak et al. found that ligand-induced EGF receptor activation results in the stimulation of tTG's crosslinking activity and causes tTG to localize to the plasma membranes of migrating cells, specifically along their leading edges. This led to studies examining whether tTG might be important for cell migration and indeed, it was found that knocking-down tTG using siRNAs or inhibiting its transamidation activity using MDC, impaired the EGF-dependent migration of HeLa cells. This then raised the question of how EGF treatment could impact tTG activity and direct its localization to leading edges. By generating HeLa cell lines stably expressing the vector only or an activated form of Ras (H-Ras G12V), Antonyak et al. were able to show that the EGF receptor signaled through Ras to direct these changes in tTG activity and localization, since tTG was constitutively activated and localized to the leading edges of the oncogenic-Ras-expressing cells. In order to determine which signaling protein was mediating signals from Ras to tTG, a number of well-known signaling networks were targeted with various inhibitors to examine the effects on tTG's activity and localization. Interestingly, of the inhibitors tested, only a c-Jun-N-terminal kinase (JNK) inhibitor was effective at blocking tTG activity and localization to the leading edges of migrating cells. While these findings argued that tTG plays an important role in EGF-driven cell migration, several questions remain regarding the mechanistic details for how EGF receptor signaling through Ras and JNK can direct these changes in tTG's activity and localization and why these changes are important for cell migration. My first data chapter will shed more light on

this process by identifying a novel mechanism regulating tTG's localization at the leading edge.

In addition to these studies, other labs have been investigating tTG for its contribution to an early step in the cancer cell migration process, namely the epithelial-to-mesenchymal transition (EMT). EMT describes a series of changes that a polarized epithelial cell undergoes to transform into a mesenchymal cell (95). This process is characterized by degradation of the cell's basement membrane which allows the cell to acquire a less-structured morphology and migrate away from its site of origin. Concurrent with this is the acquisition of more invasive characteristics, enhanced motility, and increased generation of extracellular matrix components. While EMT can be found as a normal part of development and tissue repair, it is more often associated with cancer cells as they acquire more metastatic and invasive phenotypes. In these cells, growth factor-induced signaling drives the enhanced expression of EMT-associated transcription factors including, Snail, Slug, Twist, zinc finger E-box binding homeobox 1 (Zeb1), and FoxC2. Many of these transcription factors act as transcriptional repressors of E-cadherin; thus, their increased expression results in a loss of E-cadherin expression. A "cadherin switch", whereby E-cadherin expression is lost in exchange for an increase in N-cadherin expression, is one of the hallmarks of EMT.

The investigation into tTG's role in ovarian cancer EMT and tumor metastasis began with the observation that tTG mRNA levels were increased in ovarian tumor tissues compared with surface epithelial cells from the normal ovary (96). These initial findings led researchers to examine whether tTG was important for cell adhesion and directional cell migration. Stable knock-down of tTG in SKOV3 ovarian cancer cells reduced cell adhesion and impaired their ability to migrate (50). When these cells were injected into nude mice, it was determined that

stable knock-down of tTG led to less tumor dissemination compared to cells expressing the vector alone. Thus, these findings suggest that tTG contributes to ovarian tumor metastasis.

These researchers took their work a step further showing that the reason tTG was important for metastasis was because of its role in promoting EMT (97). Specifically, ovarian cancer cells expressing tTG adopt a mesenchymal phenotype and are more invasive than their counterparts which do not express tTG. This was found to be the result of tTG activating NF-κB, which causes the transcriptional up-regulation of Zeb1, a transcriptional repressor of E-cadherin, leading to a cadherin switch and EMT. Given the links between TGF-β signaling and EMT, this group also investigated whether TGF-β could regulate tTG expression in the context of ovarian cancer. They found that secreted TGF-β did induce the expression of tTG in ovarian cancer cells, primarily through a Smad2/3-dependent mechanism, and that TGF-β treatment was sufficient to induce the EMT phenotype as read-out by decreased E-cadherin expression and increased expression of N-cadherin and Zeb1 (98). Interestingly, ovarian cancer cells which disseminate from the primary tumor to metastasize to distinct sites for secondary tumor formation form cell aggregates known as spheroids. This aggregation allows cancer cells to circulate while protecting them from environmental stresses during metastasis. These spheroids have been shown to possess characteristics similar to stem cells (sometimes called ovarian cancer initiating cells, OCIC), and express representative stem cell markers including, CD44 and CD117. To determine whether TGF-β treatment and the resulting induction of tTG expression was important for this stem cell population, investigators quantified CD44+/CD117+ populations in ovarian cancer cells which either expressed tTG for failed to express it. They found that in cells lacking tTG, the OCIC population was significantly smaller, compared with cells which express tTG. Moreover, expression levels of other stem cell-specific transcription factors

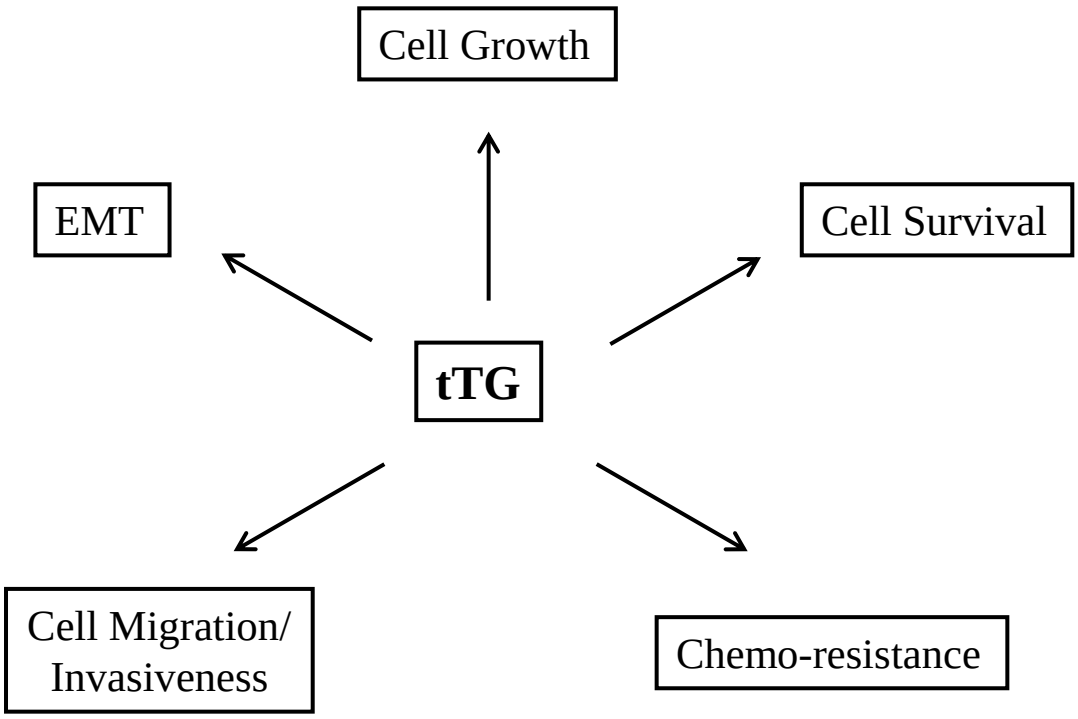
including Nanog, Oct3/4, and Sox-2 were also increased in cells with higher tTG expression. Thus, these findings demonstrate that TGF-β regulation of tTG expression may be important for driving EMT and contributing to the stem cell population which drives metastasis.

Concluding Remarks

This introduction has described much of the research performed on tTG, beginning with some of the early studies that linked increases in tTG expression and crosslinking activity to the induction of apoptosis, especially in the context of neurodegenerative diseases (19-21,33-36). These studies were followed by a series of findings that revealed tTG can also play a protective role and prevent cells from undergoing apoptosis during cellular differentiation (9,14,48). Around this time, researchers were discovering that tTG expression and/or activation were up-regulated in various types of human cancer, which led to the question: Does tTG participate in malignant transformation? We now know that tTG contributes to a number of cancer cell phenotypes including promoting cell growth, cell survival, chemo-resistance, EMT, and cell migration and invasiveness (13,27,50,52-56,61,97). All of these roles are highlighted in Figure 1.5. Studies have revealed that tTG is able to function inside the cell, at the cell membrane, or even upon its release from cells associated with microvesicles. As a result, my thesis work has focused on learning more about how tTG impacts these aspects of cancer progression, in particular cell migration and cell survival.

Chapter 2 describes work that builds upon our laboratory's initial findings that tTG is important for the EGF-dependent migration of HeLa cervical carcinoma cells, and the constitutive migration of the more aggressive breast cancer cells, MDA-MB231 cells (13). In HeLa cells, EGF treatment activates tTG and induces a change in the sub-cellular localization of tTG, resulting in its accumulation along the leading edges of actively migrating cells. In Chapter

Figure 1.5 tTG promotes several aspects of oncogenesis. To date, the research performed on tTG in the context of human cancer has revealed several roles for tTG in promoting cancer progression. tTG has been shown to regulate cell growth and survival, as well as protect cells faced with apoptotic challenges such as chemotherapeutic agents from undergoing cell death. In addition, tTG has been shown to play a key role in the acquisition of metastatic and invasive capabilities, and may facilitate EMT during this process.


Cell Growth
EMT
Cell Survival
tTG
Cell Migration/
Invasiveness
Chemo-resistance

2, I set out to learn more about how tTG is localized to leading edges and evaluate its importance for cell migration. Interestingly, we discovered that a small population (~10%) of tTG in HeLa cells is constitutively associated with the plasma membrane and that this membrane-associated population is activated and recruited to leading edges in an EGF-dependent manner. Immunoprecipitations of tTG from the membrane fractions of HeLa cells treated with EGF revealed that tTG binds members of the Hsp70 family of molecular chaperones. tTG and Hsp70 co-localize to the leading edges of migrating cells and inhibition of Hsp70's chaperonin activity specifically blocks Hsp70 and tTG from localizing to the leading edges of migrating cells and also inhibits cell migration. Thus, these findings suggest that Hsp70 facilitates tTG's localization to the leading edges of actively migrating cells and that this localization is critical for the EGF-dependent migration of HeLa cells and the constitutive migration of MDA-MB231 cells.

In Chapter 3, the focus of my work was shifted toward trying to understand how expression of tTG in a normal (non-transformed) cellular background would influence various aspects of transformation. The goal for this study was to learn more about the intrinsic ability of tTG to promote cellular transformation, versus its ability to cooperate with oncogenic proteins in cancer cells to achieve the malignant state. Using NIH3T3 fibroblasts stably expressing the vector alone or a Myc-tagged form of wild-type tTG, we found that tTG promotes cell survival by activating the PI3-kinase/mTOR/p70 S6-kinase pathway. We discovered that tTG forms a 'signaling complex' with c-Src and PI3-kinase and that inhibition of tTG or Src perturbs complex formation and prevents tTG from activating the PI3-kinase signaling pathway. Activation of this pathway is critical for tTG's ability to enhance cell viability as inhibition of any component of this pathway including Src, PI3-kinase, and mTOR, completely ablates the

protective effect of tTG against apoptosis. Thus, this study suggests that a primary role for tTG alone may be to regulate cell survival. Moreover, these findings give us reason to believe that tTG is a valid target for cancer therapy.

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CHAPTER 2

A Unique Role for Heat Shock Protein 70 and its Binding Partner Tissue Transglutaminase in Cancer Cell Migration*

Introduction

Cell migration is a highly complex, multi-step process that is carried-out by virtually all cell types at some point during their lifetime and it underlies a number of important biological outcomes that range from embryonic development to immune responses and tissue repair/regeneration (1-5). It is also a cellular function that is frequently targeted for deregulation in human cancers, as acquiring the ability to migrate aberrantly is a crucial step in the development of the invasive and metastatic phenotypes exhibited by advanced stage cancers (6,7). Although many important questions regarding how migration is regulated in different cellular contexts remain unanswered, the general process of cell migration is fairly well established. Common to all forms of directional cell migration is the formation of a polarized cell, where the asymmetric distribution of signaling proteins, phospholipids, and cytoskeletal components gives rise to a cell with both leading and trailing edges (8). The leading edge is characterized by Arp2/3-driven actin polymerization and plasma membrane protrusions which extend from the cell body in the direction of movement. These membrane protrusions then make new contact points between a cell and its underlying substrate, providing the necessary adhesion that a cell needs to move forward. However, in order for a cell to successfully migrate, immediately following the formation of new adhesion sites along the leading edge, changes at

* Published previously as Boroughs LK & Antonyak MA, et al. (2011) *J Biol Chem* 286:27094-37107.

the trailing edge of the cell must also occur. In particular, contraction of the acto-myosin cytoskeleton and the disassembly of established cell adhesion sites at the trailing edge take place in order to allow the back end of a migrating cell to detach from its substrate, retract, and then shift the remaining part of the cell body towards the leading edge.

Given the connections between cell migration and numerous biological outcomes including the progression of certain human disease states (i.e. cancer), it is not surprising that a good deal of effort has been dedicated toward understanding the mechanisms that are responsible for regulating the ability of cells to migrate. The induction of cell migration is typically triggered by growth factors and/or signals from the extracellular matrix that surrounds cells (9,10). The abilities of these extracellular stimuli to induce the activation of a series of signaling events within target cells help coordinate the extensive remodeling of the actin cytoskeleton and changes in the adhesion properties of cells that are necessary for cell migration. For example, stimulating the human cervical carcinoma cell line, HeLa, with EGF causes these cells to acquire a polarized morphology (forming both leading and trailing edges), as well as increases their ability to migrate and exhibit invasive activity (11).

Among the most extensively investigated group of signaling proteins that have been linked to EGF-induced cell migration are members of the Rho family of small GTPases including Cdc42, Rho, and Rac (12,13). Each of these highly related GTPases are regulated in a spatially and temporally specific manner in actively migrating cells. Cdc42 is necessary for establishing and maintaining cell polarity by properly positioning the nucleus and orienting microtubules in the direction of cell movement, and by helping to recruit signaling and motor/assembly proteins to the leading edges of cells. On the other hand, Rac and Rho act antagonistically toward each other, with Rac being predominantly localized to leading edges

where it promotes Arp2/3-dependent actin polymerization and the formation of cellular protrusions, whereas Rho is found along trailing edges where it participates in the contraction and bundling of the actin cytoskeleton.

While several other proteins have been implicated in EGF-stimulated cell migration, the vast majority of these are traditional signaling proteins (i.e. Ras, PI3-kinase, PLC, ERK, and JNK) (7,14,15). However, there are also a few examples of non-traditional signaling proteins that are important for certain types of cells to migrate. One such example is tTG, a dual functioning protein that combines an ability to bind and hydrolyze GTP with an enzymatic transamidation activity that generates covalent crosslinks between two proteins or between a protein and a polyamine (16,17). tTG is over-expressed in a significant percentage of advanced stage and high grade human cervical, lung, brain, prostate, and breast tumors, and its transamidation (crosslinking) activity has been shown to be essential for the invasive/metastatic behavior of highly aggressive cancer cells, such as the MDAMB231 human breast cancer cell line (18-21). Recently, we showed that the stimulation of HeLa carcinoma cells with EGF resulted in tTG activation and its accumulation at their leading edges, whereas knock-downs of tTG, or exposure of the cells to the tTG inhibitor monodansylcadaverine (MDC), inhibited the EGF-stimulated migration and invasive activity of these cells (11). Although these findings demonstrate a fundamental role for tTG, and in particular its enzymatic crosslinking activity, in EGF-stimulated cancer cell migration, what remains to be determined is how EGF triggers the accumulation of tTG at the leading edges of cells and whether this event is important for the ability of tTG to promote cell migration.

In this study, we have taken an important step toward answering these questions by uncovering a novel connection between tTG, a component of the chaperonin network, Hsp70,

and the ability of human cancer cells to migrate. We identify Hsp70 as a novel tTG-interacting partner, and show that the ability of plasma membrane-associated tTG to localize to the leading edges of EGF-stimulated HeLa cells, as well as to the leading edges of constitutively migrating MDAMB231 breast cancer cells, is sensitive to inhibition of Hsp70. Importantly, we further demonstrate that exposure of these different cell lines to inhibitors of the ATP hydrolytic activity of Hsp70, while having no effect on the protein crosslinking activity of tTG, inhibits EGF-induced HeLa cell migration and the constitutive migration normally exhibited by MDAMB231 breast carcinoma cells. To our knowledge, these findings show for the first time that the chaperonin capabilities of heat shock proteins can participate in cell migration by helping to target key regulatory proteins (i.e. tTG) to the leading edges of migrating cells, as well as demonstrate that the proper localization of tTG to leading edges is crucial for its ability to promote cancer cell migration.

Results

tTG is recruited to the leading edges of migrating cancer cells

The accumulating evidence implicating tTG as an important contributor to cancer cell migration, invasion, and metastasis (11,19,20) prompted us to investigate further the possible regulatory mechanisms that underly its ability to promote cell migration. We chose the HeLa cervical carcinoma cell line as our initial model system because we had previously established that stimulating HeLa cells with EGF induced tTG activation and showed that knocking-down tTG, or treatment of these cells with the tTG crosslinking inhibitor MDC, blocked their EGF-stimulated migration and invasive activity (11). Using scratch or wound-healing assays as a means to read-out the rates of cell migration, we confirmed the importance of tTG activity in

mediating EGF-stimulated HeLa cell migration by showing that the ability of EGF to promote the migration of HeLa cells, as indicated by their movement into the wound, is severely ablated when the cells are treated with MDC (Figure 2.1A, *compare the last two images in the top panels*). Likewise, knocking-down tTG in HeLa cells using two different siRNAs (Figure 2.1B, *left panels*) inhibited the EGF-dependent migration of these cells (Figure 2.1B, *right panels*). However, we also noticed by immunofluorescent analysis that treating serum-starved HeLa cells with EGF for increasing lengths of time caused a detectable change in the subcellular distribution of tTG (Figure 2.1C, *top row on the left*). While in untreated HeLa cells, tTG was expressed primarily throughout the cytoplasm, EGF treatment of the cells for as little as 1 hour caused an accumulation of tTG at their plasma membranes, particularly along leading edges, as indicated by its co-localization with sites of F-actin build-up (Figure 2.1C, *middle row*), as well as with the leading edge marker cortactin (22) (Figure 2.1D, *left panels*). This effect of EGF on the subcellular localization of tTG persisted even after 12 hours of EGF stimulation (Figure 2.1C, *top row*), suggesting that tTG could be a leading edge-resident protein. Consistent with this idea, immunofluorescence performed on serum-starved MDAMB231 breast carcinoma cells, a highly invasive/metastatic human cancer cell line whose constitutive migration capabilities are dependent on tTG as determined by MDC treatment (Figure 2.1A, *compare the last two images in the bottom panels*) and by tTG knock-down (Figure 2.1E), revealed that tTG accumulated along their leading edges as well (Figure 2.1C, *on the right*, and Figure 2.1D, *on the right*). Taken together, these findings raise the intriguing possibility that the recruitment of tTG to the leading edges of actively migrating cells may represent an important regulatory step underlying its ability to promote cell migration.

Figure 2.1 tTG is localized to the leading edges of actively migrating cells and its crosslinking activity is necessary for cell migration. (**A**) Scratch assays were performed on HeLa cells (*top panels*) and MDAMB231 cells (*bottom panels*) treated without (*Untreated*) or with EGF, and without or with MDC, as indicated. MDAMB231 cells were fixed 12 hours after striking the wound; HeLa cells were fixed after 24 hours. The cells were then visualized using light microscopy and the extent of wound closure determined. One set of untreated cells was fixed immediately after striking the wound (*Untreated 0 hr.*) to indicate the size of the initial wounds. The widths of the initial wounds are indicated by dashed lines. (**B**) The extracts collected from HeLa cells transfected with control-RNAi, tTG-RNAi 1, or tTG-RNAi 2 were immunoblotted with tTG and actin antibodies (*left panels*). Scratch assays were then performed on cells transfected with the same siRNAs, treated without (*Untreated*) or with EGF. The cells were processed as outlined in *A* (*right panels*). (**C and D**) Duplicate sets of serum-starved cultures of HeLa cells and MDAMB231 cells were treated without (*Untreated*) or with EGF for increasing lengths of time, as indicated, and then were fixed. (**C**) Immunofluorescence was performed on one set of the cells using a tTG antibody, rhodamine-conjugated phalloidin (*Actin*), and DAPI (to stain nuclei). (**D**) Immunofluorescence was performed on the second set of cells using tTG and cortactin antibodies and DAPI. Representative fluorescent images of the cells are shown and the localization of tTG and cortactin at leading edges is indicated with arrows. (**E**) The extracts collected from MDAMB231 cells transfected with control-RNAi, tTG-RNAi 1, or tTG-RNAi 2 were immunoblotted with tTG and actin antibodies (*top panels*). Scratch assays were then performed on cells transfected with the same siRNAs and the cells were processed as outlined in *A* (*bottom panels*).

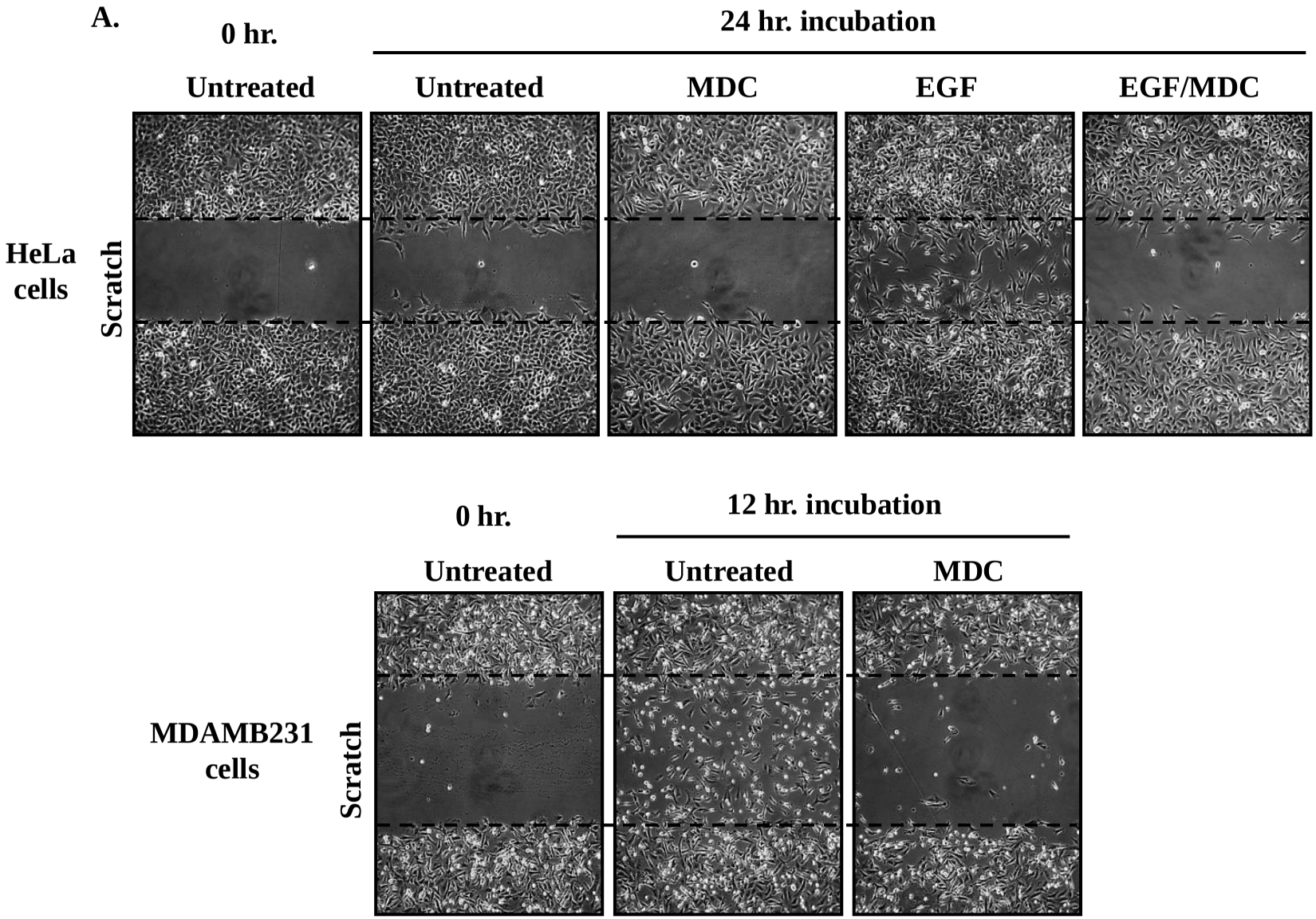

A.
0 hr.
24 hr. incubation
Untreated
Untreated
MDC
EGF
EGF/MDC
HeLa cells
Scratch
0 hr.
12 hr. incubation
Untreated
Untreated
MDC
MDAMB231 cells
Scratch

Figure 2.1 (Continued)

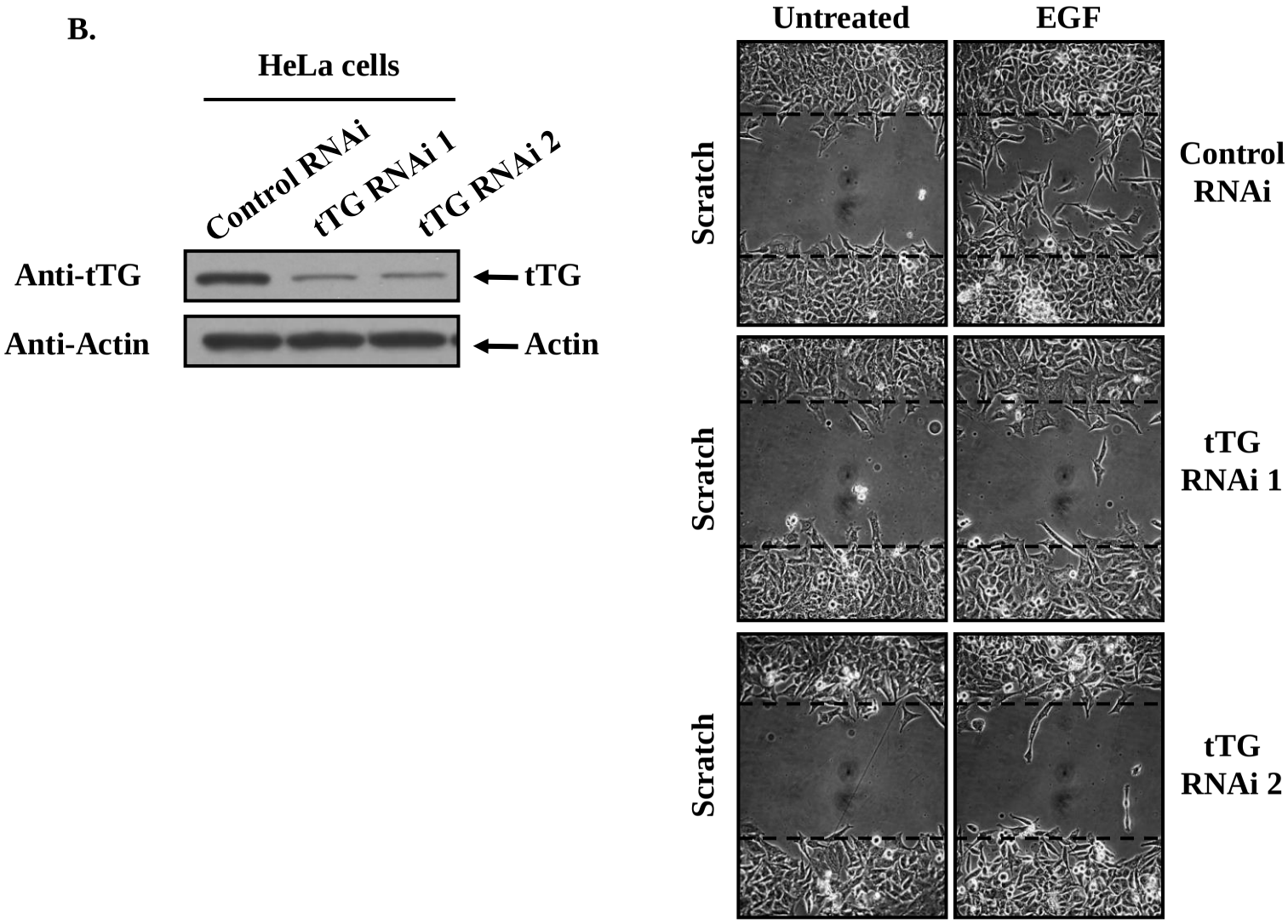


Figure 2.1 (Continued)

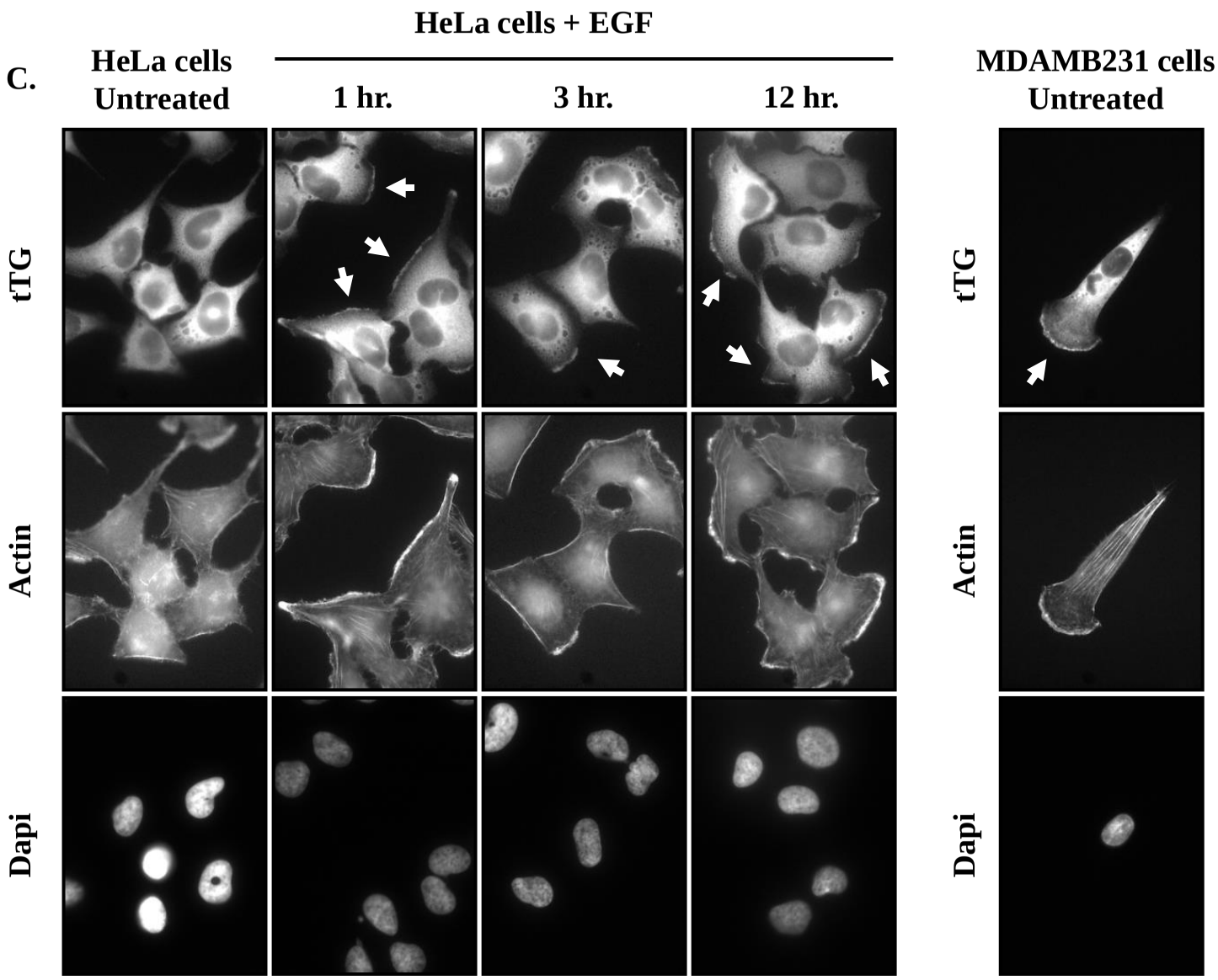


Figure 2.1 (Continued)

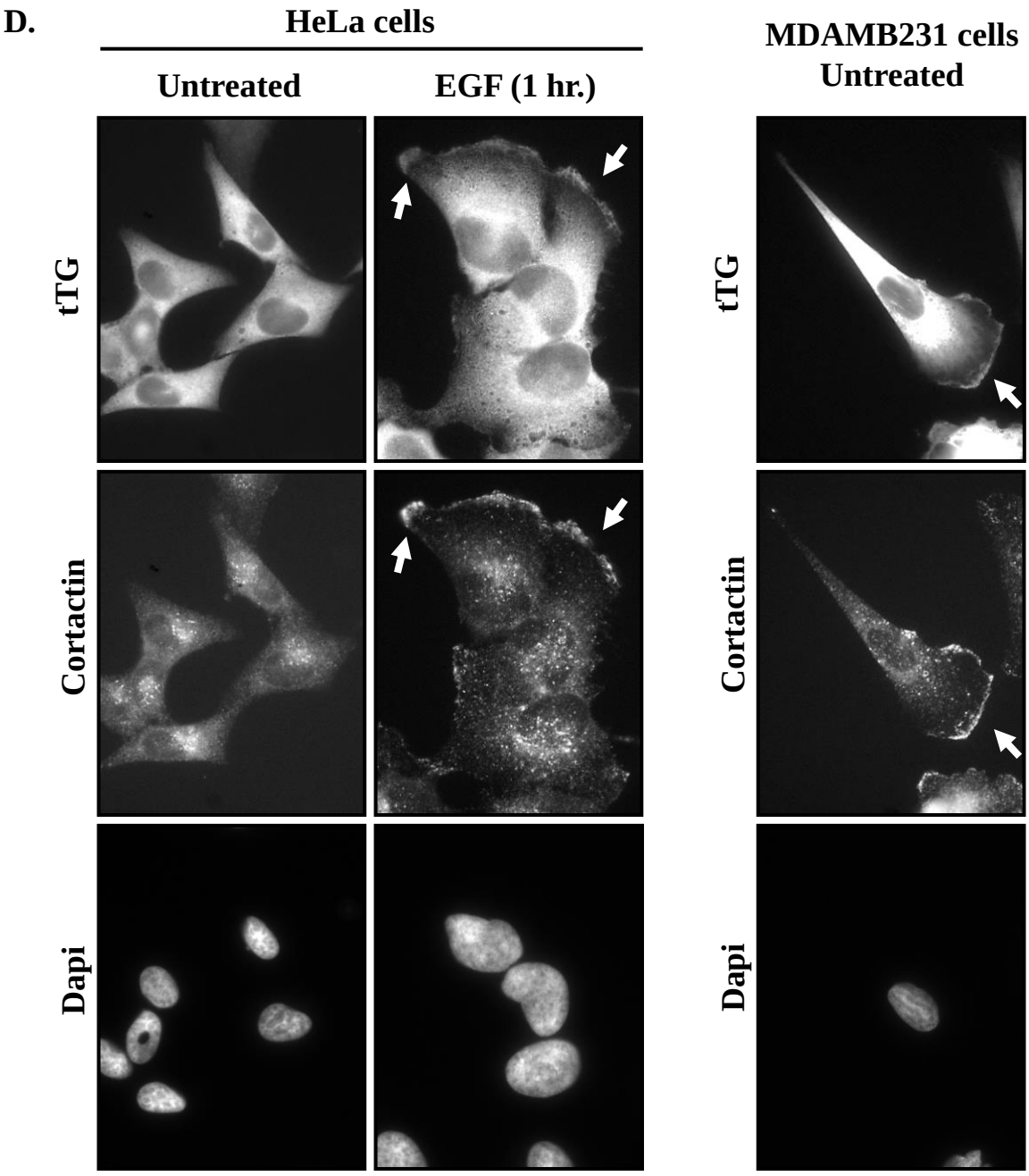
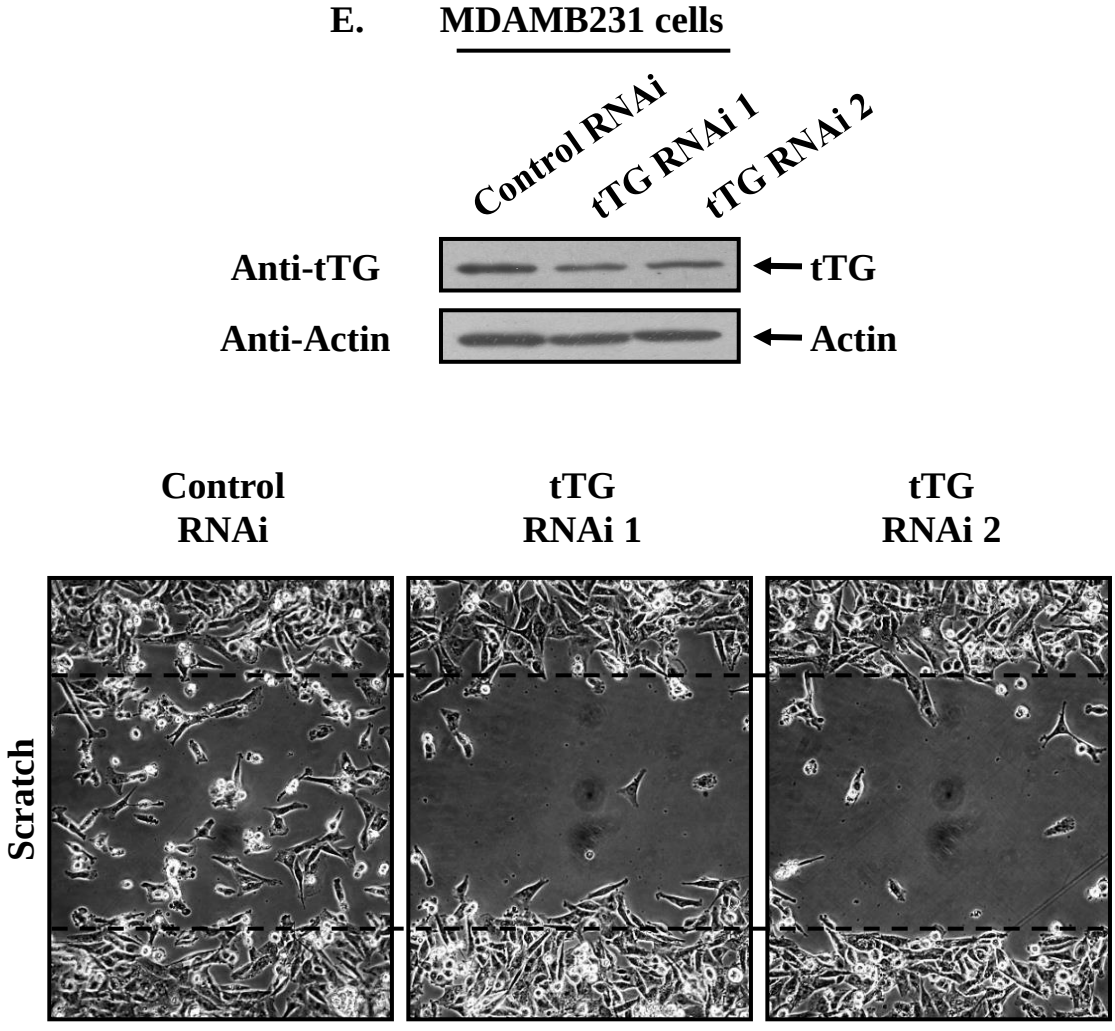


Figure 2.1 (Continued)



A plasma membrane-associated pool of tTG is trafficked to leading edges

In order to determine whether the localization of tTG at leading edges plays an essential role in cell migration, we set out to better understand how tTG accumulates at these distinct regions of the plasma membrane. Based on our immunofluorescence experiments (Figures 2.1C and 2.1D) it appears that tTG is predominantly expressed in the cytoplasm of untreated HeLa cells, but can then be detected along their leading edges in response to EGF stimulation. Thus, we initially suspected that the appearance of tTG at the leading edges of EGF-stimulated HeLa cells was an outcome of its recruitment from the cytoplasm to the plasma membrane. To determine whether this was indeed the case, we examined whether the changes in the distribution of tTG in HeLa cells treated with EGF, as observed by immunofluorescence, could also be detected using a cell fractionation approach (i.e. as monitored by increases in the amount of tTG present in the membrane fractions collected from EGF-stimulated HeLa cells). Serum-starved HeLa cells that had been left untreated, or were stimulated with EGF for 1, 3, or 12 hours, were collected, divided into their cytosolic and membrane fractions, and the resulting extracts were immunoblotted. Figure 2.2A shows that the fractionation procedure was successful, as indicated by the enrichment of the plasma membrane-associated protein, fibronectin, in the membrane fractions, and the cytosolic protein, IκBα, in the cytoplasmic fractions (Figure 2.2A, *second and third panels from the top, respectively*). However, when the blot was probed for tTG, we obtained a surprising result. Rather than detecting increased amounts of tTG in the membrane fractions from the EGF-stimulated HeLa cells compared to those collected from untreated HeLa cells, we found that the levels of tTG were essentially equivalent (determined by densitometry to be 10 % ± 2.5 % of the tTG expressed in the cells) in these different membrane fractions (Figure

Figure 2.2 A pool of tTG is constitutively associated with the plasma membrane. Serum-starved HeLa cells that had been treated without (*0*) or with EGF for increasing lengths of time, as indicated, were homogenized and then subjected to differential centrifugation to separate cytosolic and membrane fractions. (**A**) The cellular fractions were immunoblotted with tTG, fibronectin, IκBα, and actin antibodies. (**B**) The same membrane fractions were also assayed for tTG transamidation activity by determining the incorporation of BPA into lysate proteins. (**C**) Actively growing HeLa cells that had been either mock transfected without DNA (*Mock*) or transfected with various Myc-tagged forms of tTG including wild-type (*tTG WT*), a transamidation-defective mutant (*tTG C277V*), or a GTP-binding-defective mutant (*tTG R580K*), were homogenized and then subjected to differential centrifugation to isolate cytosolic and membrane fractions. The fractions were immunoblotted with Myc, fibronectin, IκBα, and actin antibodies. (**D**) Synthetic liposomes were prepared by extrusion and then equal amounts of this preparation were combined with either 5 μg recombinant tTG (*tTG WT*) or 5 μg bovine serum albumin (*BSA*). After a 15 minute incubation, the liposomes were pelleted by centrifugation, and the resulting supernatant (*Sup*) and liposome (*Pellet*) fractions were resolved by SDS-PAGE. The gel was then stained with Quick Blue to detect the proteins. A lane containing recombinant tTG (*Rec. tTG WT*) was included as a standard.

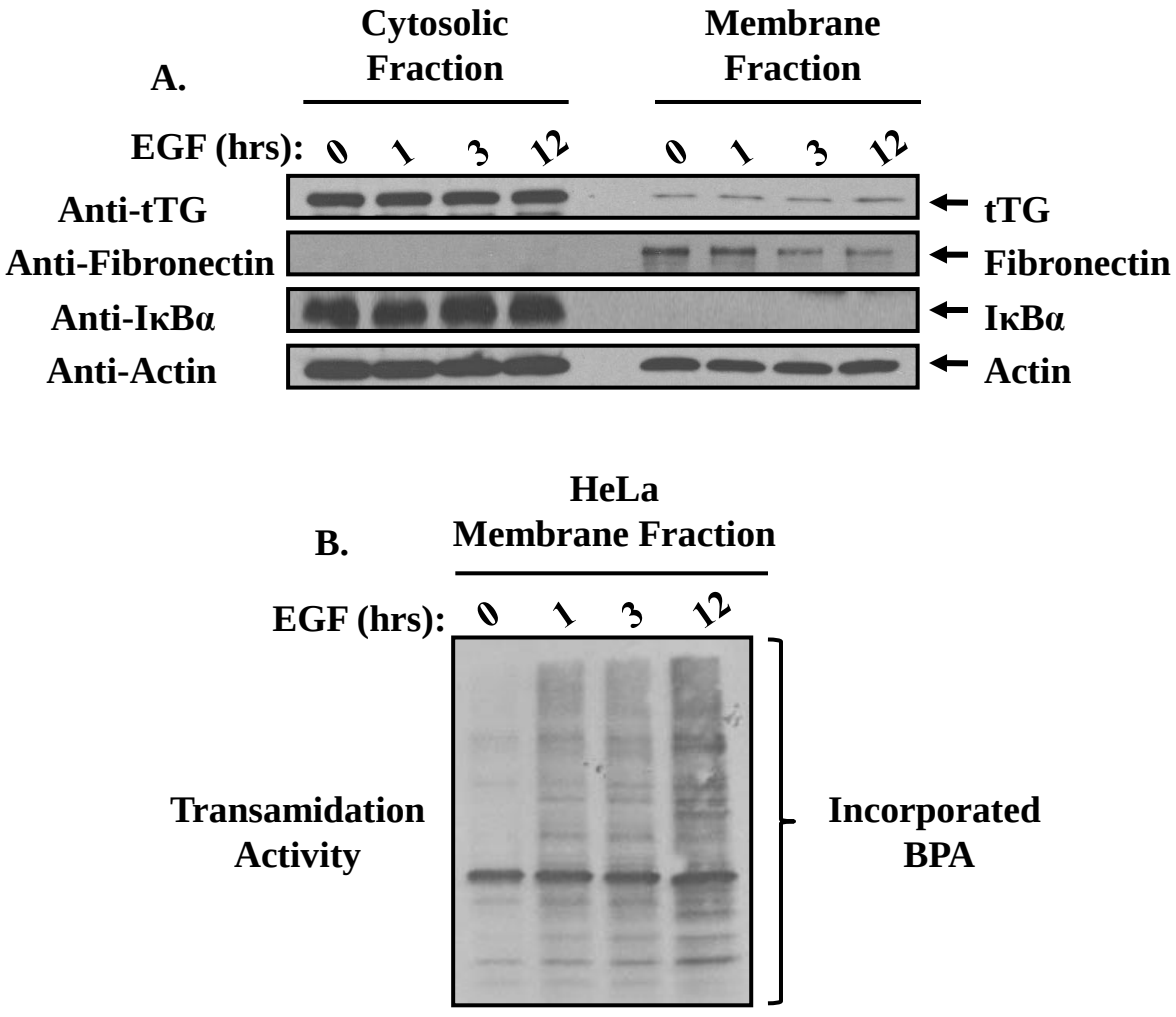
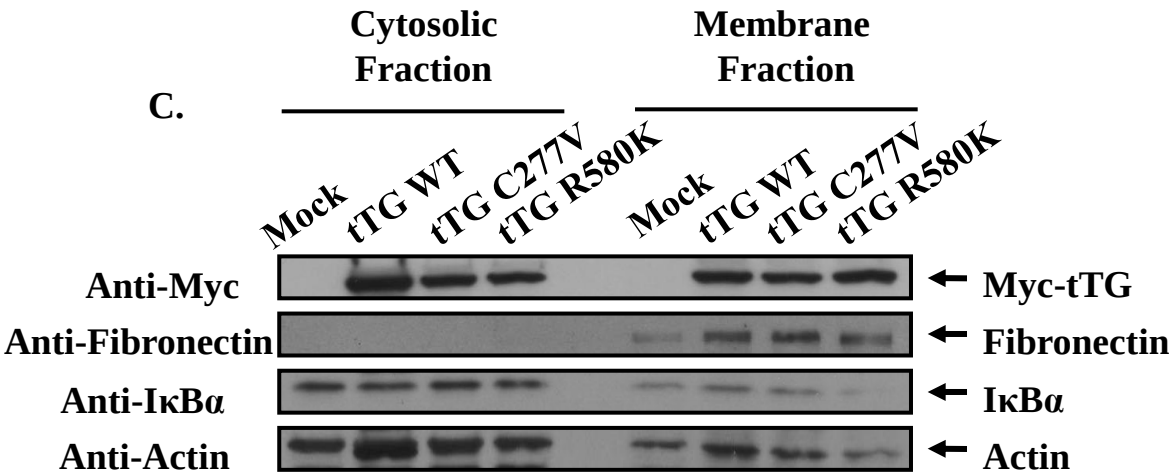
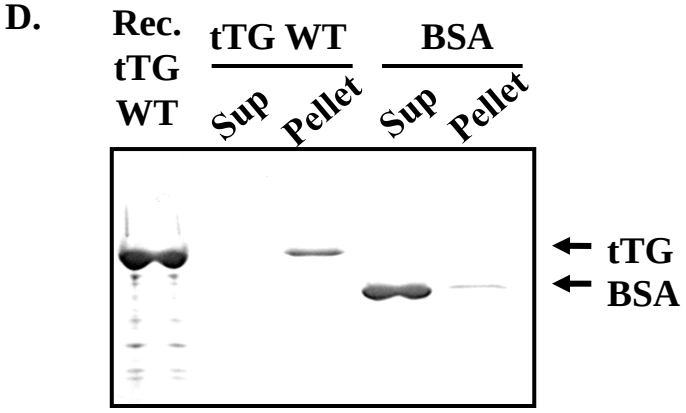

A.
Cytosolic Fraction
Membrane Fraction
EGF (hrs): 0 1 3 12 0 1 3 12
Anti-tTG
tTG
Anti-Fibronectin
Fibronectin
Anti-IκBα
IκBα
Anti-Actin
Actin
B.
HeLa Membrane Fraction
EGF (hrs): 0 1 3 12
Transamidation Activity
Incorporated BPA

Figure 2.2 (Continued)





2.2A, *top panel*). This indicated that EGF does not direct the translocation of tTG from the cytoplasm to the surface of HeLa cells. Instead, it appears that a small but discrete pool of tTG associates with the plasma membrane in serum-starved HeLa cells (that cannot be detected by immunofluorescence), and is redistributed in an EGF-dependent manner such that it accumulates at the leading edges of cells.

We next examined whether EGF treatment would affect the activity of the plasma membrane-associated pool of tTG. The same membrane fractions analyzed in Figure 2.2A were assayed for tTG-catalyzed transamidation activity, as read-out by the incorporation of BPA into lysate proteins. Figure 2.2B shows that little tTG activity was detectable in the membrane fraction collected from serum-starved HeLa cells. In contrast, EGF stimulation caused a marked increase in the crosslinking activity of the plasma membrane-associated tTG. The increase in tTG activity was detectable within 1 hour of EGF treatment and was maintained, if not slightly enhanced, through 12 hours of continuous EGF stimulation. It is also worth noting that the ability of EGF to stimulate the enzymatic crosslinking activity of the membrane-associated tTG, and to cause tTG to accumulate at the leading edges of HeLa cells, occur on similar time-scales (*compare Figure 2.1C, left panel, and Figure 2.2B*), highlighting the fact that both the activation and the localization of tTG to the leading edges of cells are tightly coupled EGF-dependent signaling events.

To gain a better understanding for how tTG associates with the plasma membrane, two additional experiments were performed. In the first experiment, we examined whether the GTP-binding capability or the transamidation activity of tTG was required for it to associate with plasma membranes. Actively growing HeLa cells ectopically expressing Myc-tagged forms of wild-type tTG (tTG WT), or mutant forms of tTG that were defective in their abilities to either

catalyze the formation of protein crosslinks (tTG C277V) or to bind GTP (tTG R580K) (23,24), were collected and fractionated into their cytosolic and membrane components. The resulting lysates were then immunoblotted with a Myc antibody to detect the different ectopically expressed forms of tTG. The top panel in Figure 2.2C shows that the ectopically expressed transamidation-defective form of tTG (*tTG C277V*), and the GTP-binding-defective mutant (*tTG R580K*), were localized to the membrane fractions from HeLa cells just as efficiently as the ectopically expressed wild-type tTG, indicating that the ability of tTG to associate with plasma membranes does not require its GTP-binding or transamidation activity.

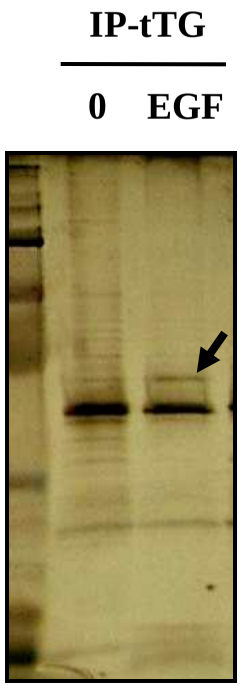
In the second experiment, we examined whether tTG could directly associate with plasma membranes or if it requires additional proteins to do so. To address this question, we used an *in vitro* approach similar to one that was developed to examine the ability of the small GTPase Cdc42 to interact with synthetically-derived liposomes (25). Purified recombinant wild-type tTG (tTG WT), or bovine serum albumin (BSA) serving as a control, was combined together with synthetically-derived liposomes whose lipid composition was similar to that of the inner leaflet of the plasma membrane in mammalian cells. Following a brief incubation, the vesicles were pelleted by centrifugation, and then the resulting supernatants and pellets were resolved by SDS-PAGE and stained with Quick Blue to detect the proteins. Figure 2.2D shows that tTG has a relatively high affinity for lipids, as nearly all of the recombinant tTG (*tTG WT*) was found to have pelleted with the synthetic vesicles. On the other hand, bovine serum albumin (*BSA*) only weakly pelleted with the liposomes, with most of the control protein remaining in the supernatant.

Hsp70 is a novel tTG-interacting protein

The findings above describe the identification and initial characterization of a plasma membrane-associated pool of tTG that may have important consequences for cell migration. However, they did not shed light on how tTG accumulates at the leading edges of migrating cells. In particular, we wondered whether tTG may need to interact with another protein(s) to achieve this localization. Thus, we set out to identify proteins that interacted with tTG in the plasma membranes from EGF-stimulated HeLa cells by mass spectrometry. Serum-starved cultures of HeLa cells that had been left untreated or were stimulated with EGF for 12 hours (i.e. a time period that we knew to be sufficient for the accumulation of tTG at the leading edges of cells) were collected and their corresponding membrane fractions isolated and then subjected to immunoprecipitation using an anti-tTG antibody. The resulting immunocomplexes were analyzed by SDS-PAGE followed by Colloidal Blue staining to detect proteins that co-immunoprecipitated with tTG. Whereas several proteins co-immunoprecipitated with tTG from the membrane fractions prepared from untreated HeLa cells, the majority of these proteins were not detected when tTG was immunoprecipitated from the membrane fractions derived from EGF-stimulated cells (Figure 2.3A). A notable exception was a protein band with an apparent molecular mass of ~70 kDa (Figure 2.3A, *arrow)*. Since we were searching for proteins that would interact with tTG in EGF-stimulated cells, as candidates for helping to mediate the translocation of tTG to leading edges, we determined the identity of the ~70 kDa protein by mass spectrometry. In fact, we found that this protein band consisted of two highly homologous and functionally redundant members of the heat shock protein family, namely Hsp70 and Hsc70 (26) (Figure 2.3B). What makes the identification of Hsc70 and Hsp70 as novel interacting partners for plasma membrane-associated tTG particularly intriguing is that they were recently shown to

Figure 2.3 Hsp70 interacts with plasma membrane-associated tTG. (**A**) Serum-starved HeLa cells that had been treated without (*0*) or with EGF for 12 hours were homogenized and then subjected to differential centrifugation to isolate the membrane components of the cells. Immunoprecipitations with a tTG antibody (*IP:tTG*) were performed on the membrane extracts and the resulting immunocomplexes were resolved by SDS-PAGE. The gel was then stained with Colloidal Blue to detect the proteins that co-immunoprecipitated with tTG. One protein band (M_r ~70 kDa), denoted with an arrow, was determined to contain two isoforms of the heat shock protein 70 family, Hsp70 and Hsc70, by mass spectrometry. (**B**) The protein sequences of human Hsp70 and Hsc70 are shown in gray. The peptide fragments identified by mass spectrometry are shaded in black. (**C**) Immunoprecipitations with a Myc antibody were performed on the extracts of HeLa cells ectopically expressing V5-tagged Hsc70 and Myc-tagged tTG, treated without (*Untreated*) or with EGF. The whole cells lysates (*WCL*) and the resulting immunocomplexes (*IP:Myc*) were immunoblotted with V5 and Myc antibodies. Non-specific mouse IgG control antibody and beads-only control immunoprecipitations were also performed on the extracts to show that the Hsc70-tTG interaction was specific. (**D**) Immunoprecipitations with a Myc antibody were performed on the extracts of actively growing HeLa (*top panels*) and MDAMB231 cells (*bottom panels*) that were ectopically expressing V5-tagged Hsc70 and a Myc-tagged form of either wild-type tTG (*tTG WT*) or a transamidation-defective form of tTG (*tTG C277V*). The whole cell lysates (*WCL*) and the resulting immunocomplexes (*IP:Myc*) were immunoblotted with V5 and Myc antibodies. Non-specific mouse IgG control antibody and beads-only control immunoprecipitations were performed on the extracts collected from HeLa and MDAMB231 cells expressing V5-Hsc70 and Myc-tTG WT to show that the Hsc70-tTG interaction was specific.

A.



B.

Hsp70

MAKAAAVGIDLGTTYSCVGVFQHGK**VEIIANDQGNRTTPSYVAFTDTER**LIGDAAK**NQVALNPQNTVF**
DAKRLIGRKFGDPVVQSDMKHWPFQVINDGDKPKVQVSYKGETKAFYPEEISSMVLTKMKEIAEAYLGY
PVTNAVITVPAYFNDSQRQATK**DAGVIAGLNVLRIINEPTAAAIAYGLDR**TGKGERNVLIFDLGGGTFDV
SILTIDDGIFEVKATAGDTHLGGEDFDNRLVNHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSST
QASLEIDSLFEGIDFYTSITRARFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDLVLVGGSTRIPKVQKLLQ
DFFNGRDLNKSINPDEAVGYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLETAGGVMTALIKRNSTIP
TKQTQIFTTYSDNQPGVLIQVYEGERAMTKDNNLLGRFELSGIPPAPGVPQIEVTFDIDANGILNVTATDKS
TGKANKITITNDKGRLSKEEIERMVQEAEKYKAEDEVQRERVSAKNALESYAFNMKSAVEDEGLKGKISE
ADKKKVLDKCQEVISWLDANTLAEKDEFEHKRKELEQVCNPIISGLYQGAGGPGPGGFGAQGPKGGSGS
GPTIEEVD

Hsc70

MSKGPAVGIDLGTTYSCVGVFQHGK**VEIIANDQGNRTTPSYVAFTDTER**LIGDAAK**NQVAMNPTNTVF**
DAKRLIGRRFDDAVVQSDMKHWPFMVVNDAGRPK**VQVEYKGETK**SFYPEEVSSMVLTKMKEIAEAYL
GK**TVTNAVVTVPAYFNDSQR**QATKDAGTIAGLNVLR**IINEPTAAAIAYGLDKK**VGAERNVLIFDLGGGT
FDVSILTIEDGIFEVKSTAGDTHLGGEDFDNRMVNHFIAEFKRKHKKDISENKRAVRRLRTACERAKRTLS
SSTQASIEIDSLYEGIDFYTSITRARFEELNADLFRGTLDPVEKALRDAKLDK**SQIHDIVLVGGSTR**IPKIQK
LLQDFFNGKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIKRNT
TIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGILNVSA
VDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDEKQRDKVSSK**NSLESYAFNMK**ATVEDEKLQ
GKINDEDKQKILDKCNEIINWLDKNQTAEKEEFEHQQKELEKVCNPIITKLYQSAGGMPGGMPGGFPGGG
APPSGGASSGPTIEEVD

C.

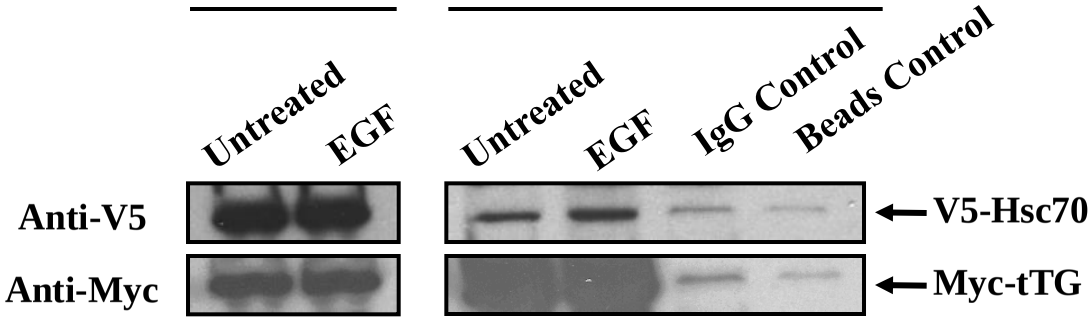
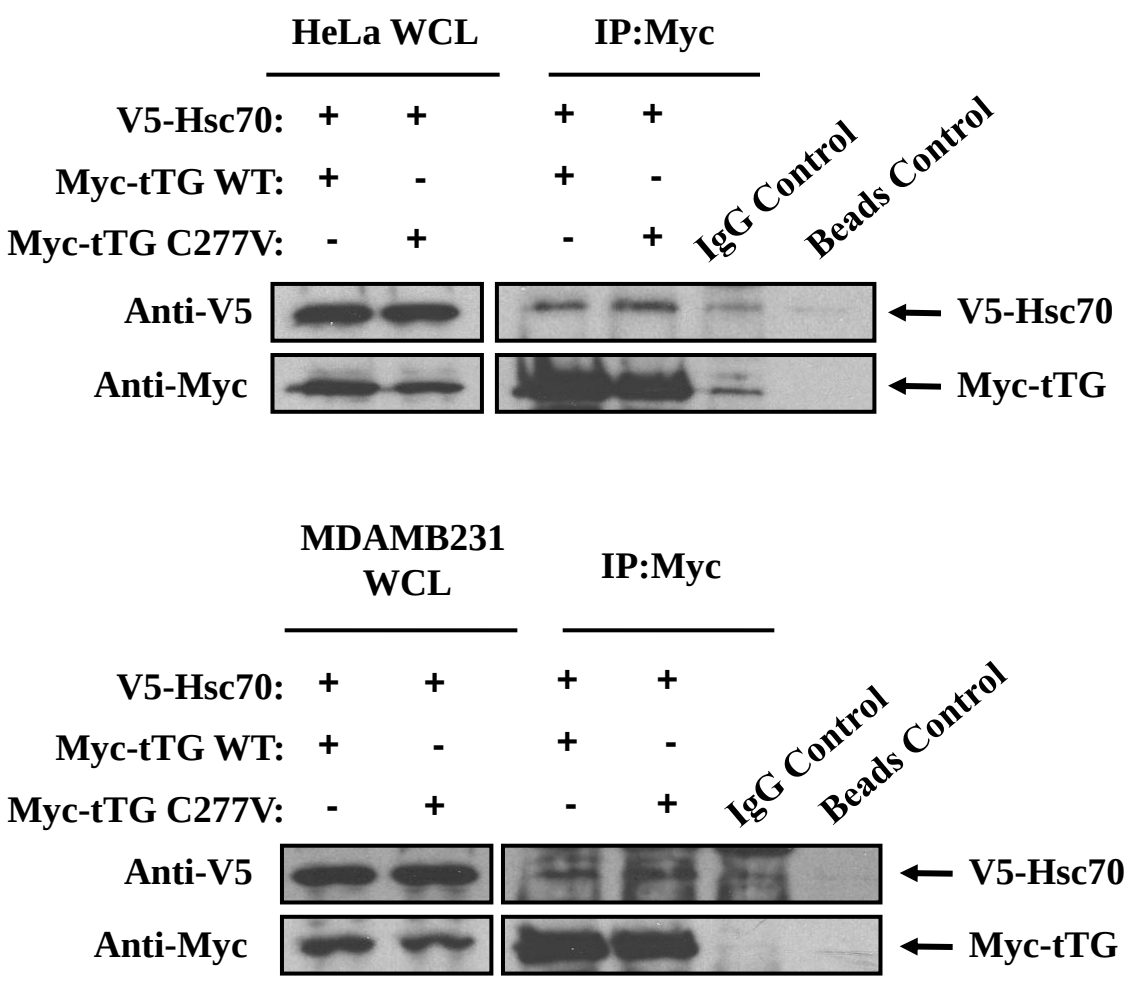


Figure 2.3 (Continued)

D.



be uniquely capable of integrating into artificial lipid bilayers, as well as into plasma membranes derived from cancer cell lines (27,28). We first confirmed this interaction by performing immunoprecipitations with a Myc antibody on the cellular lysates from HeLa cells transiently co-expressing a V5-tagged form of Hsc70 and a Myc-tagged form of tTG, treated without or with EGF. Figure 2.3C shows that V5-tagged Hsc70 can be co-immunoprecipitated with Myc-tagged tTG, independent of EGF treatment. Next we compared the ability of Hsc70 to interact with wild-type tTG and a transamidation defective form of tTG (C277V) by performing immunoprecipitations with a Myc antibody on the cellular extracts from HeLa cells (Figure 2.3D, *top panels*), as well as MDAMB231 breast cancer cells (Figure 2.3D, *bottom panels*), that were transiently co-expressing a V5-tagged form of Hsc70 and a Myc-tagged form of tTG. Figure 2.3D shows that V5-tagged Hsc70 can be co-immunoprecipitated with Myc-tagged tTG (*Myc-tTG WT*) in each of these cell types. Moreover, the transamidation-defective form of tTG (*Myc-tTG C277V*), when ectopically expressed in HeLa cells or MDAMB231 cells, was able to bind Hsc70 as efficiently as wild-type tTG, suggesting that the ability of tTG to interact with heat shock protein family members does not require its enzymatic transamidation activity.

We next used immunofluorescence approaches to determine whether Hsp70, like tTG, was expressed along the leading edges of migrating cells. Our reasoning was that if the binding of tTG to Hsp70 family members is necessary for the recruitment and accumulation of tTG at leading edges, then Hsp70 and tTG should be present together at these cellular sites. Cultures of serum-starved HeLa cells co-expressing V5-tagged Hsc70 and Myc-tagged tTG, that were either untreated or stimulated with EGF, were fixed and then stained with Myc and V5 antibodies. The resulting fluorescent images showed that the V5-tagged Hsc70 was predominantly cytoplasmic in the serum-starved HeLa cells (Figure 2.4A, *left panels*). However, following EGF-

Figure 2.4 tTG and Hsp70 co-localize to the leading edges of cells. (**A**) HeLa and MDAMB231 cells ectopically expressing V5-Hsc70 and Myc-tTG were fixed and then subjected to immunofluorescence using V5 and Myc antibodies and DAPI (to stain nuclei). Representative images of the transfectants are shown, with the co-localization of the ectopically expressed forms of tTG and Hsc70 at leading edges being highlighted with arrows. (**B**) Serum-starved HeLa cells and MDAMB231 cells were treated without (*Untreated*) or with EGF for 12 hours, as indicated, and fixed. Immunofluorescence was performed on the cells using tTG and Hsp70 antibodies and DAPI. Representative images of the cells are shown, with the co-localization of tTG and Hsp70 at leading edges being indicated with arrows.

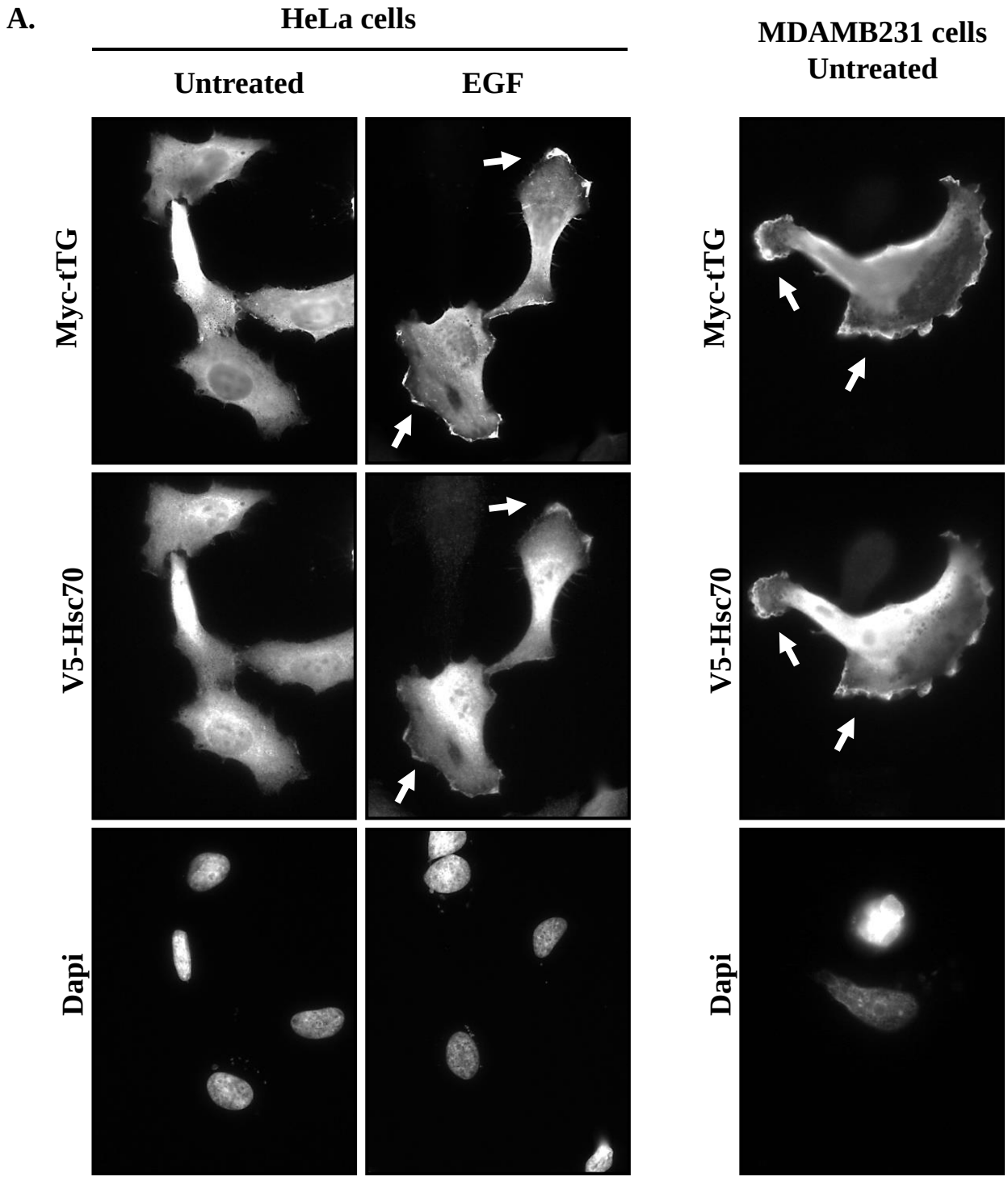
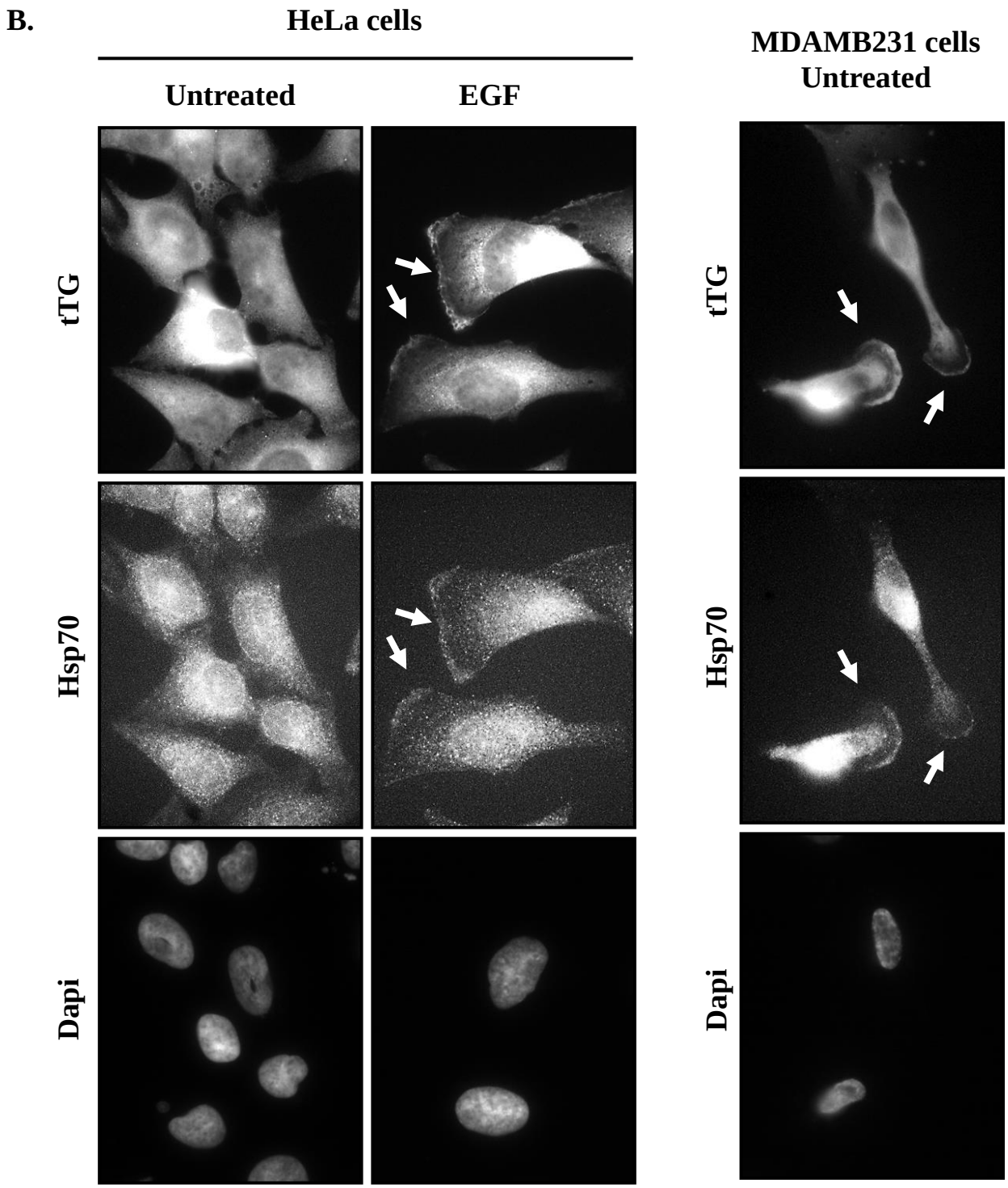

A.
HeLa cells
Untreated
EGF
MDAMB231 cells
Untreated
Myc-tTG
V5-Hsc70
Dapi
Myc-tTG
V5-Hsc70
Dapi

Figure 2.4 (Continued)



stimulation, the V5-tagged form of Hsc70 was readily detectable at the leading edges of these cells, where it co-localized with the ectopically expressed form of tTG (*Myc-tTG WT*).

An analogous experiment was then performed on the constitutively migrating MDAMB231 breast cancer cell line. Consistent with the findings from HeLa cells, immunofluorescent analysis performed on MDAMB231 cells co-expressing V5-tagged Hsc70 and Myc-tagged tTG showed that both of these ectopically expressed proteins co-localized along the leading edges of these cells (Figure 2.4A, *right panels*). Endogenous Hsp70 was also shown to consistently localize to the developing leading edges in EGF-treated HeLa cells and in MDAMB231 cells (Figure 2.4B, *left and right panels, respectively*).

Plasma membrane-associated tTG is recruited to leading edges by Hsp70

We next set out to determine whether Hsp70 was responsible for the accumulation of tTG at the leading edge. Initially we tried to examine this by knocking-down Hsp70 family members from cells and seeing whether the ability of tTG to be localized to leading edges was disrupted. However, despite a number of attempts to knock-down Hsp70 and Hsc70, either individually or in combination, in HeLa cells or MDAMB231 cells (including our use of 6 different siRNAs targeting Hsp70 and/or Hsc70 in varying amounts for transfection), we have thus far been unable to significantly reduce their expression levels (*data not shown*). Consequently, we turned to three different inhibitors of the ATP-hydrolytic activity of Hsp70, namely, myricetin, methylene blue, and VER 155008, to investigate the involvement of Hsp70 in targeting tTG to leading edges. Figure 2.5A shows that the exposure of EGF-stimulated HeLa cells to any of these inhibitors was sufficient to block the ability of tTG, as well as Hsp70, to accumulate along leading edges. The results of these experiments are quantified in Figure 2.5B, and show that the

Figure 2.5 Inhibiting Hsp70 activity blocks the ability of tTG and Hsp70 to localize at leading edges. (**A**) Serum-deprived HeLa cells were treated without (*Untreated*) or with EGF, -/+ myricetin, methylene blue, or VER 155008, and then immunofluorescence was performed on the cells using tTG and Hsp70 antibodies. The resulting fluorescent images are shown with the tTG and Hsp70 at leading edges being indicated with arrows. (**B**) Quantification of the cells shown in *A* with tTG at their leading edges. (**C**) Serum-starved MDAMB231 cells were treated without (*Untreated*) or with myricetin, methylene blue, or VER 155008, and then immunofluorescence was performed on the cells using tTG and Hsp70 antibodies.. The resulting fluorescent images are shown with the tTG and Hsp70 at leading edges being indicated with arrows. (**D**) Quantification of the cells shown in *C* with tTG at their leading edges. Three independent experiments were performed, with at least 250 cells being scored for each condition. The results from the experiments were then averaged together and graphed. The error bars indicate standard deviation.

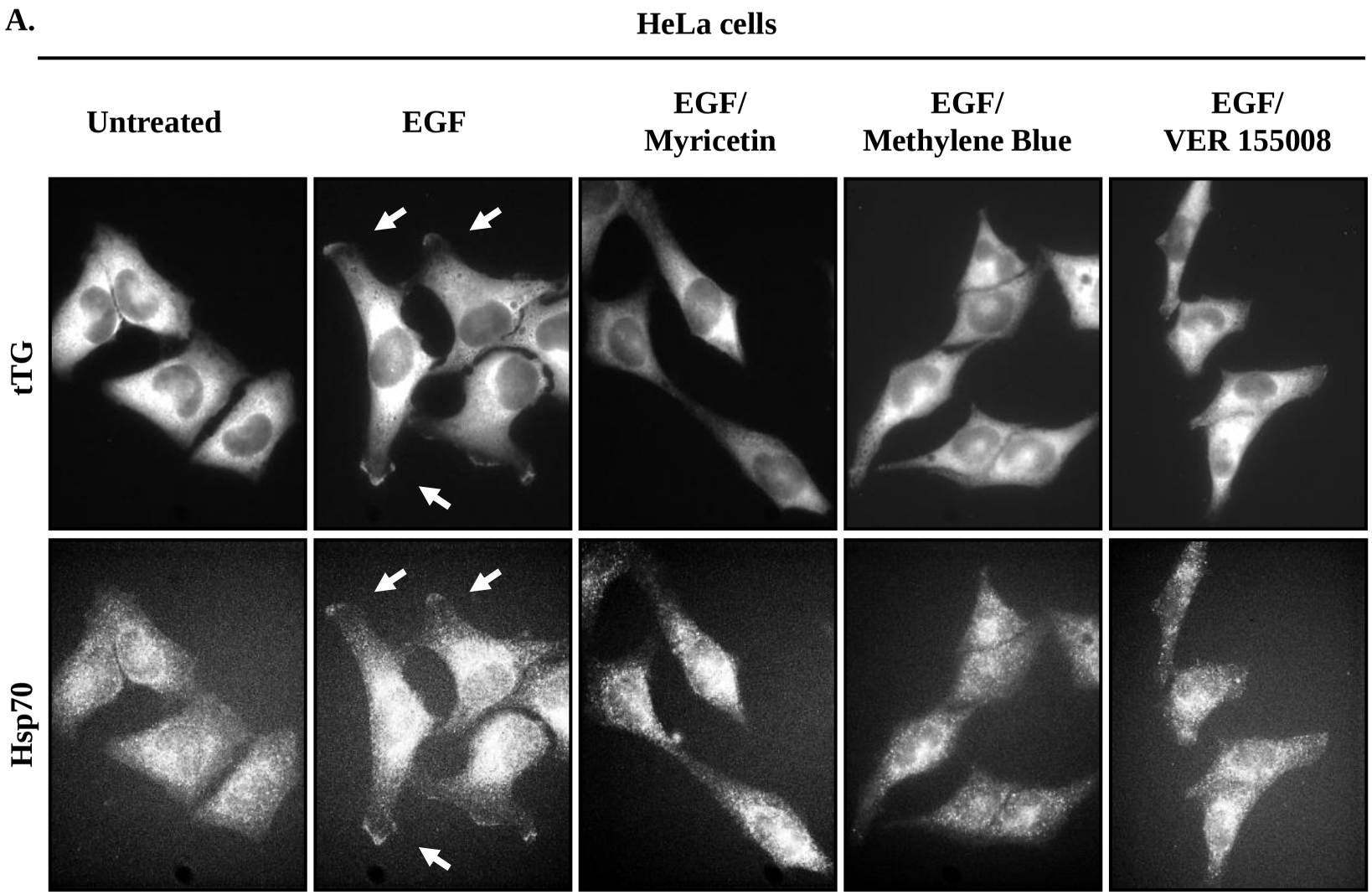

A.
HeLa cells
Untreated
EGF
EGF/
Myricetin
EGF/
Methylene Blue
EGF/
VER 155008
tTG
Hsp70

Figure 2.5 (Continued)

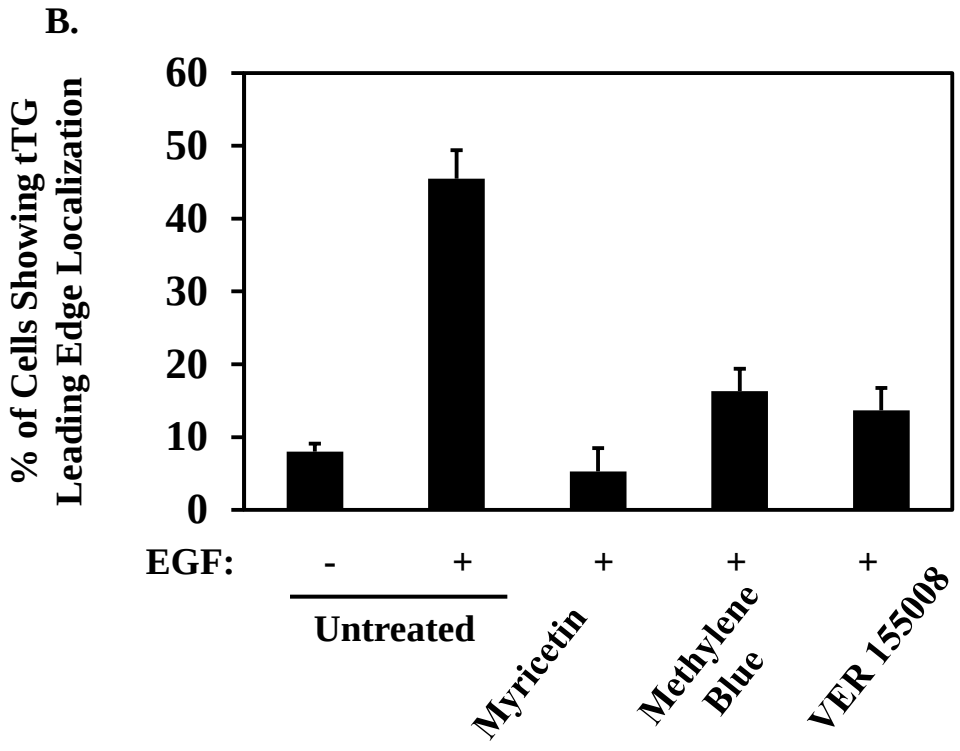
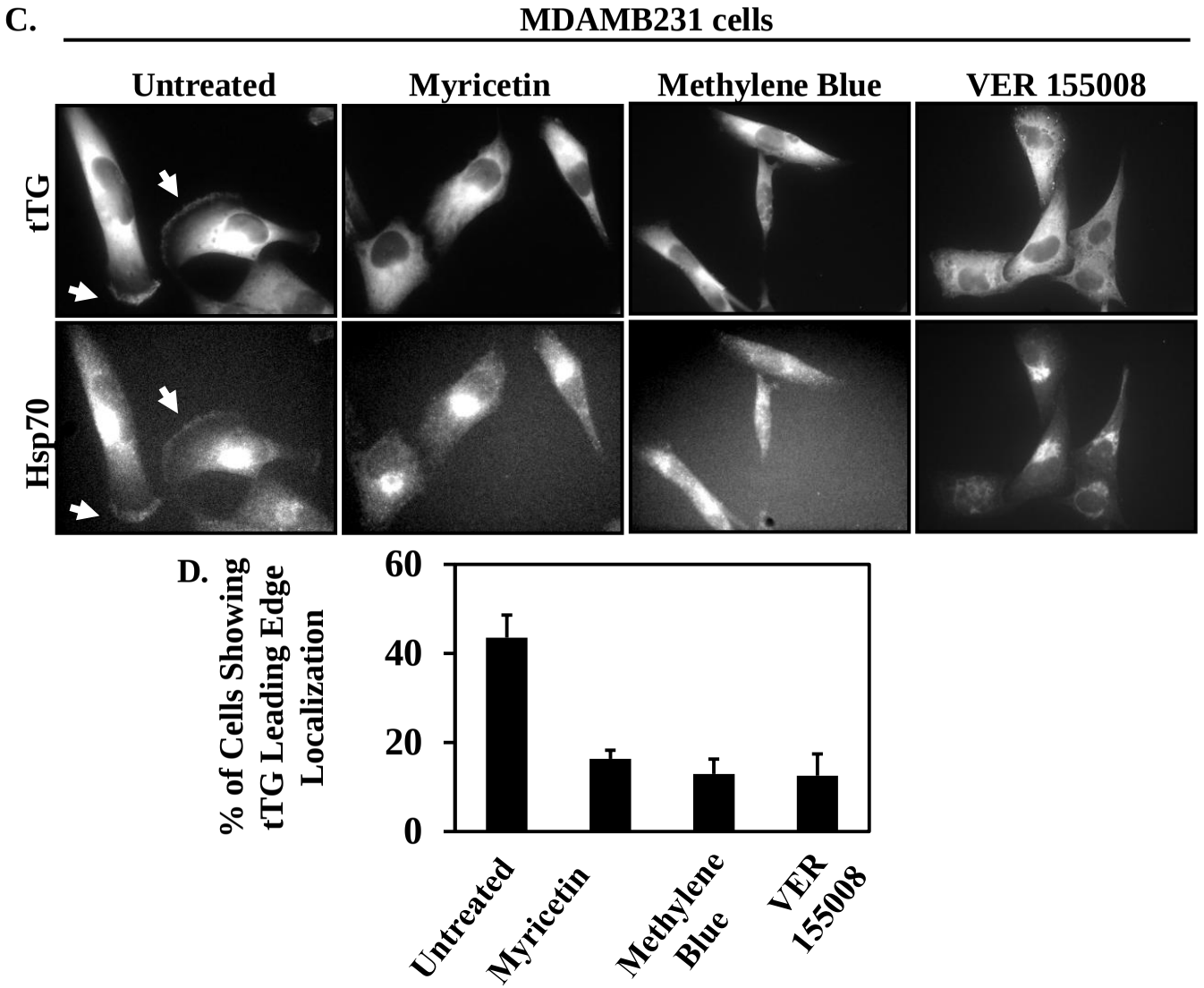


Figure 2.5 (Continued)



Hsp70 inhibitors reduced the percentage of HeLa cells with tTG along their leading edges from ~40% to between ~5-15%, depending on which inhibitor was used. Hsp70 inhibition also blocked the leading edge accumulation of tTG in MDAMB231 breast cancer cells (Figure 2.5C), with the results of these experiments being quantified in Figure 2.5D, indicating that Hsp70 activity is essential for redistributing the plasma membrane-associated pool of tTG to leading edges in at least two different cell types. Moreover, it is worth emphasizing that the effect of the three Hsp70 inhibitors at preventing the recruitment of tTG to leading edges appears to be specific, and not simply a consequence of disrupting leading edge formation in cells, based on two additional pieces of data. First, the characteristic build-up of actin that occurs at the leading edges of EGF-stimulated HeLa cells or in constitutively migrating MDAMB231 cells, that is necessary to promote membrane protrusions and the generation of new cell-to-substrate contacts, was not inhibited when the cells were exposed to either myricetin, methylene blue, or VER 155008 (Figures 2.6A and 2.6B, *respectively*). Second, we also found that myricetin treatment did not disrupt the ability of two ectopically expressed, activated forms of the leading edge-resident proteins, Rac (HA-tagged Rac F28L) and Ras (HA-tagged Ras G12V), to be localized to these sites in MDAMB231 cells (Figure 2.6C).

The ATP-hydrolytic activity of heat shock proteins is essential for their ability to associate with client proteins (29). While traditionally the binding of heat shock proteins to client proteins occurs in response to cellular stresses, such as elevated temperatures, as a means to help ensure proper protein folding under stressful conditions, more recently, heat shock proteins have been implicated in promoting human cancer progression (30,31). Not only are the expression and activation levels of several heat shock proteins, including Hsp70, frequently up-regulated in a variety of primary tumors and tumor-derived cell lines, but the ability of heat

Figure 2.6 Inhibition of Hsp70 does not have a global effect on leading edge proteins. (**A**) Serum-deprived HeLa cells were treated without (*Untreated*) or with EGF, -/+ myricetin, methylene blue, or VER 155008, and then immunofluorescence was performed on the cells using a tTG antibody, rhodamine-conjugated phalloidin (*Actin*), and DAPI. The resulting fluorescent images are shown with the tTG at leading edges being indicated with arrows. (**B**) Serum-starved MDAMB231 cells were treated without (*Untreated*) or with myricetin, methylene blue, or VER 155008, and then immunofluorescence was performed on the cells using a tTG antibody, rhodamine-conjugated phalloidin (*Actin*) and DAPI. The resulting fluorescent images are shown with the tTG at leading edges being indicated with arrows. (**C**) MDAMB231 cells were transiently transfected with either of two HA-tagged constructs: constitutively active Rac (F28L) or GTP hydrolysis-defective Ras (G12V), and immunofluorescence was performed using HA and tTG antibodies and DAPI. The resulting fluorescent images are shown with the Rac, Ras, and tTG at leading edges being indicated with arrows.

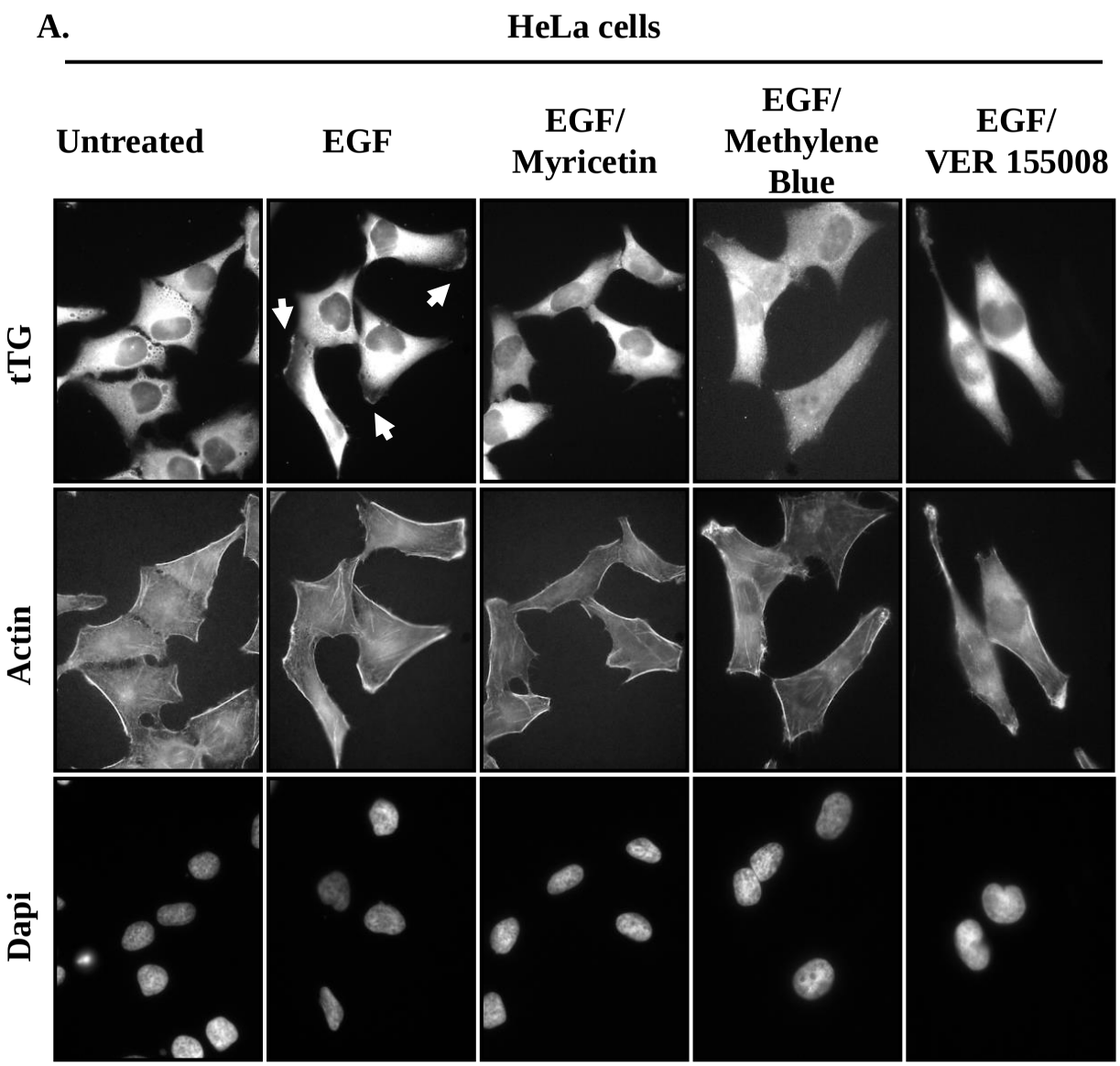

A.
HeLa cells
Untreated
EGF
EGF/ Myricetin
EGF/ Methylene Blue
EGF/ VER 155008
tTG
Actin
Dapi

Figure 2.6 (Continued)

B. **MDAMB231 cells**

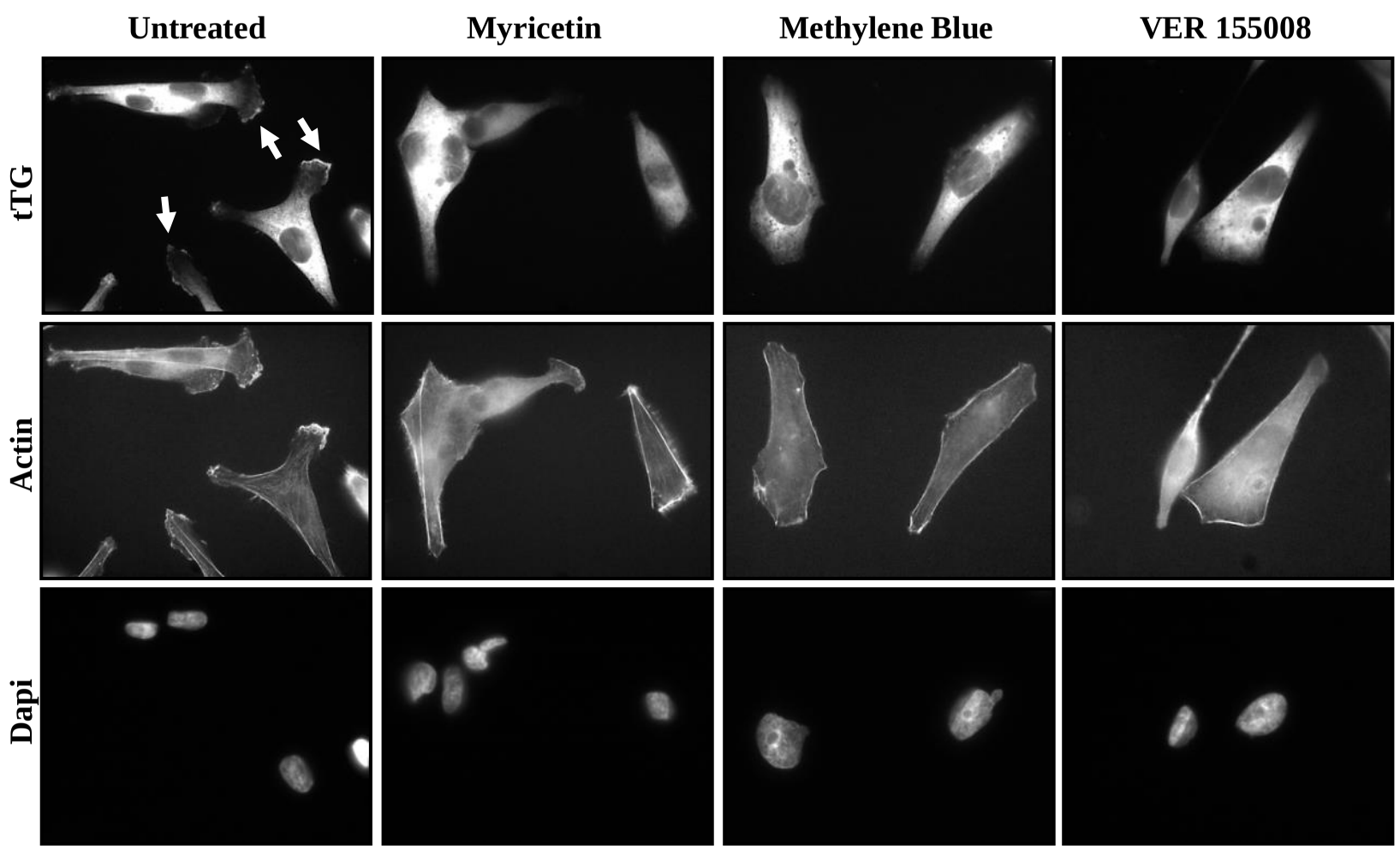
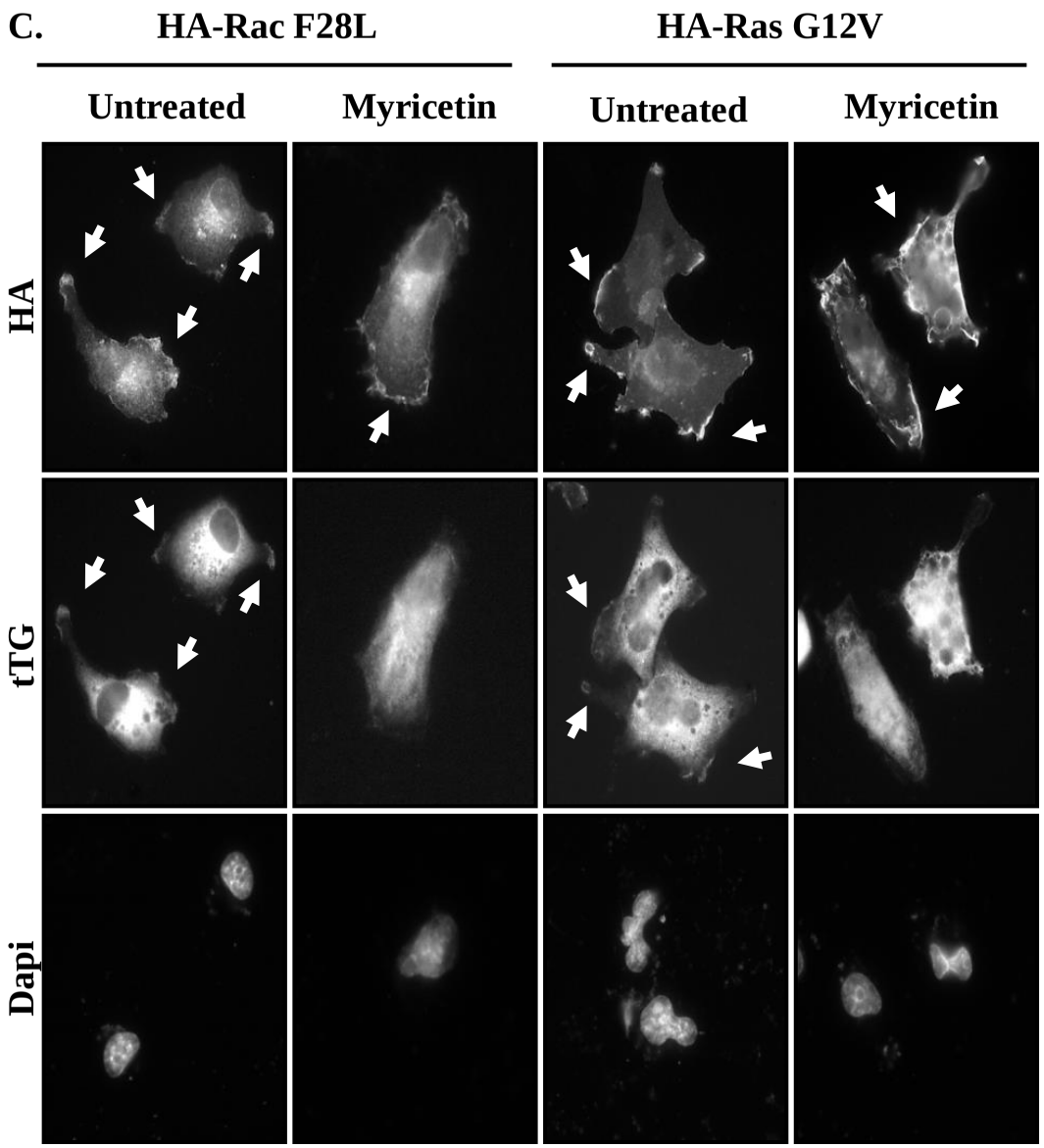


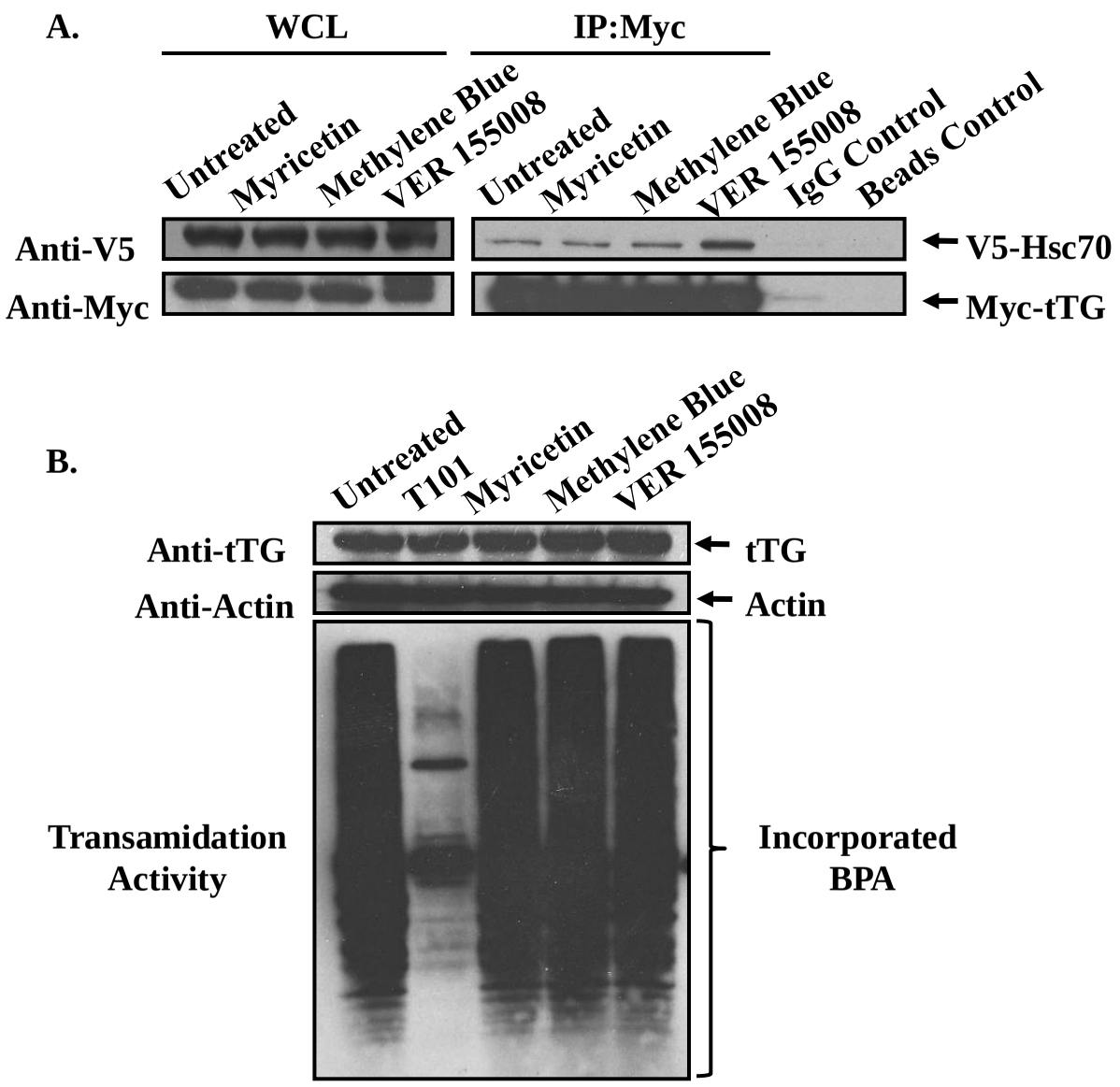
Figure 2.6 (Continued)



shock proteins to bind to and potentiate the signaling capabilities of key mitogenic and pro-survival signaling proteins has been shown to be necessary for inducing/maintaining the transformed state (30,32). Given our novel finding that the recruitment of tTG to the leading edges of HeLa cells and MDAMB231 cells is dependent on Hsp70 activity, it was logical to consider the possibility that tTG might be a client of Hsp70. If this were the case, then we would predict that the interaction between Hsp70 and tTG in cells would be sensitive to treatment with myricetin, methylene blue, or VER 155008, since these inhibitors function by blocking the ATP-hydrolytic activity of Hsp70, and thereby disrupt its ability to bind client proteins through the conserved substrate (client) binding domain. HeLa cells transiently co-expressing V5-tagged Hsc70 and Myc-tagged tTG were either left untreated or were incubated with one of the inhibitors and lysed. The cell extracts were then subjected to immunoprecipitation using a Myc antibody, with the resulting immunocomplexes then analyzed by Western blot. Figure 2.7A shows that V5-tagged Hsc70 co-immunoprecipitated with Myc-tagged tTG from each of the inhibitor-treated cells to a similar extent as it did from the untreated (control) HeLa cells. This suggests that tTG is not a client of Hsp70 in the conventional sense and does not directly bind to Hsp70 through its substrate (client) binding site.

Next, we asked how inhibiting Hsp70 would affect tTG activity. Serum-starved MDAMB231 cells that had been left untreated, or were incubated with an irreversible tTG crosslinking inhibitor, T101, or with myricetin, methylene blue, or VER 155008, were collected and then the enzymatic transamidation activities in each sample were assayed by reading-out the incorporation of BPA into lysate proteins. Consistent with previous results, tTG expressed in serum-starved MDAMB231 cells was constitutively active, while as expected, treatment of the cells with the tTG inhibitor, T101, reduced tTG activity (Figure 2.7B). However, the amount of

Figure 2.7 Inhibiting the ATP-hydrolytic activity of Hsp70 has no effect on the interaction between tTG and Hsp70, or on the protein crosslinking activity of tTG. (**A**) HeLa cells ectopically expressing V5-Hsc70 and Myc-tTG were treated without (*Untreated*) or with myricetin, methylene blue, or VER 155008, as indicated, and lysed. Immunoprecipitations with a Myc antibody were performed on the cell extracts, followed by SDS-PAGE and Western blot analysis using V5 and Myc antibodies. Non-specific mouse IgG control antibody and beads-only control immunoprecipitations were performed on the extracts from the untreated cells to show that the Hsc70-tTG interaction was specific. (**B**) Serum-starved cultures of MDAMB231 cells were treated without (*Untreated*) or with T101, myricetin, methylene blue, or VER 155008 and then lysed. The extracts were immunoblotted with tTG and actin antibodies (*top panels*), and assayed for transamidation activity as read-out by the incorporation of BPA into lysate proteins (*bottom panel*).


A.
WCL
IP:Myc
Untreated
Myricetin
Methylene Blue
VER 155008
Untreated
Myricetin
Methylene Blue
VER 155008
IgG Control
Beads Control
Anti-V5
Anti-Myc
V5-Hsc70
Myc-tTG
B.
Untreated
T101
Myricetin
Methylene Blue
VER 155008
Anti-tTG
Anti-Actin
tTG
Actin
Transamidation Activity
Incorporated BPA

tTG-catalyzed transamidation activity detected in the inhibitor-treated MDAMB231 cell samples was similar to that detected in the control sample.

The recruitment of tTG to leading edges is important for cell migration

Our findings showing that inhibiting Hsp70 activity prevented the accumulation of tTG at the leading edges of cells, without affecting its transamidation activity, afforded us with a unique opportunity to examine the importance of tTG's localization to leading edges on the ability of cells to migrate. Multiple plates of confluent HeLa cells or MDAMB231 cells, that either were untreated or incubated with different combinations of EGF and myricetin, were subjected to a scratch (or wound healing) assay, and their rates of cell migration were compared. The top panels in Figure 2.8A show that the EGF-stimulated migration of HeLa cells, as indicated by the ability of these cells to close the wound, was blocked when the cells were also cultured in the presence of myricetin. Likewise, the constitutive migration activity normally exhibited by the MDAMB231 cells was also ablated by myricetin treatment (Figure 2.8A, *bottom panels*). Similar results were obtained using the two additional Hsp70 inhibitors, methylene blue (Figure 2.8B) and VER 155008 (Figure 2.8C). Taken together, these findings demonstrate that the proper localization of tTG to leading edges by Hsp70 family members is essential for the migration of certain human cancer cell lines.

Discussion

Cell migration is a fundamental process in biology that underlies key stages of development and tissue regeneration; however, it is frequently deregulated in human diseases such as cancer, where the aberrant migration of cells serves as a precursor to their metastatic and

Figure 2.8 Inhibition of Hsp70 blocks the migration of HeLa and MDAMB231 cells. (**A**) Scratch assays were performed on serum-deprived HeLa cells treated without (*Untreated*) or with EGF, -/+ myricetin, and on serum-starved MDAMB231 cells -/+ myricetin. The MDAMB231 cells were fixed 12 hours after striking the wound; HeLa cells were fixed after 24 hours. The cells were then visualized using light microscopy and the extent of wound closure determined. One set of untreated cells was fixed immediately after striking the wound (*Untreated 0 hr.*) to indicate the size of the initial wounds. The widths of the initial wounds are indicated by dashed lines. (**B**) Scratch assays were performed on the cell lines using methylene blue as outlined in *A*. (**C**) Scratch assays were performed on the cell lines using VER 155008 as outlined in *A*.

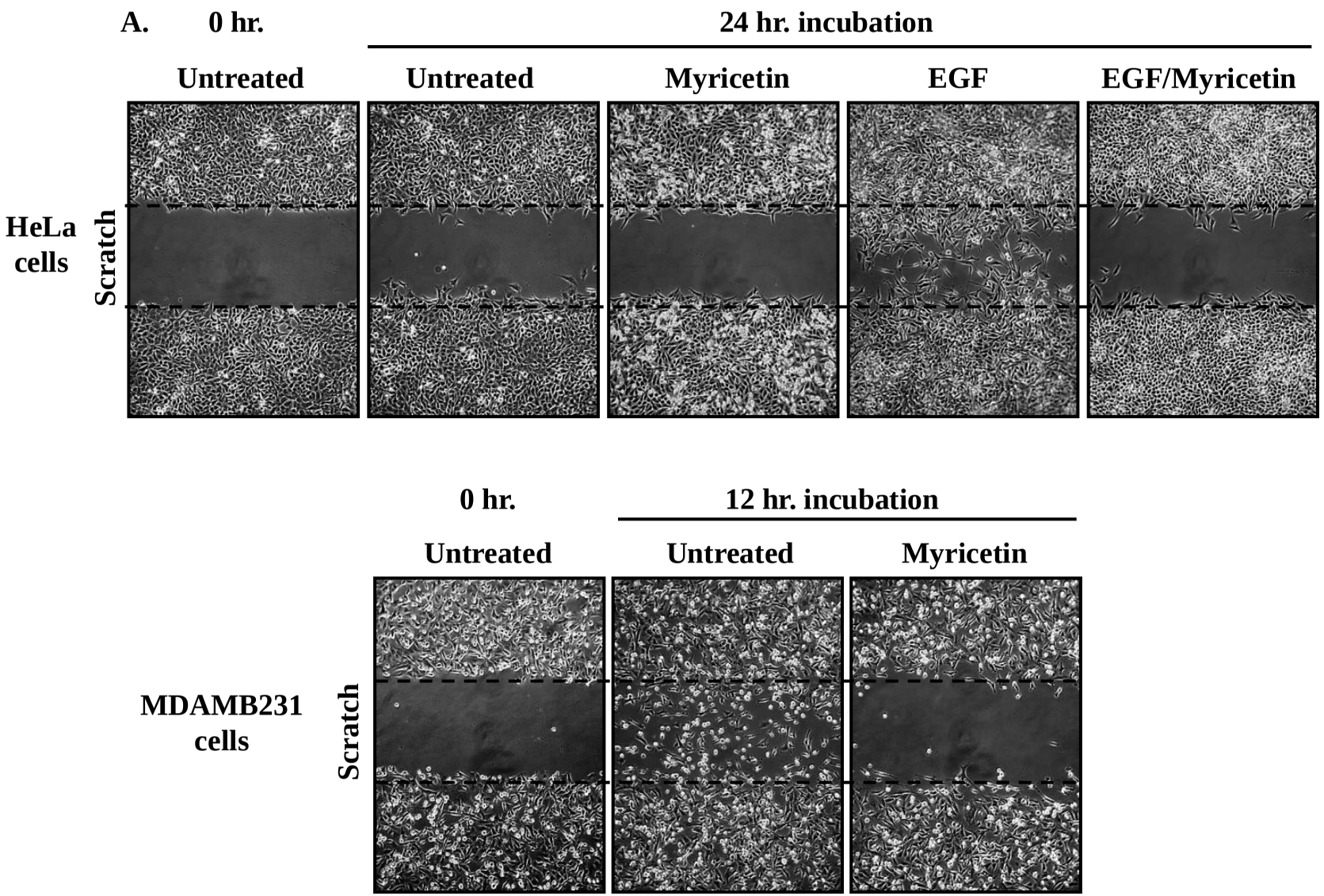

A.
0 hr.
24 hr. incubation
Untreated
Untreated
Myricetin
EGF
EGF/Myricetin
HeLa cells
Scratch
0 hr.
12 hr. incubation
Untreated
Untreated
Myricetin
MDAMB231 cells
Scratch

Figure 2.8 (Continued)

B.

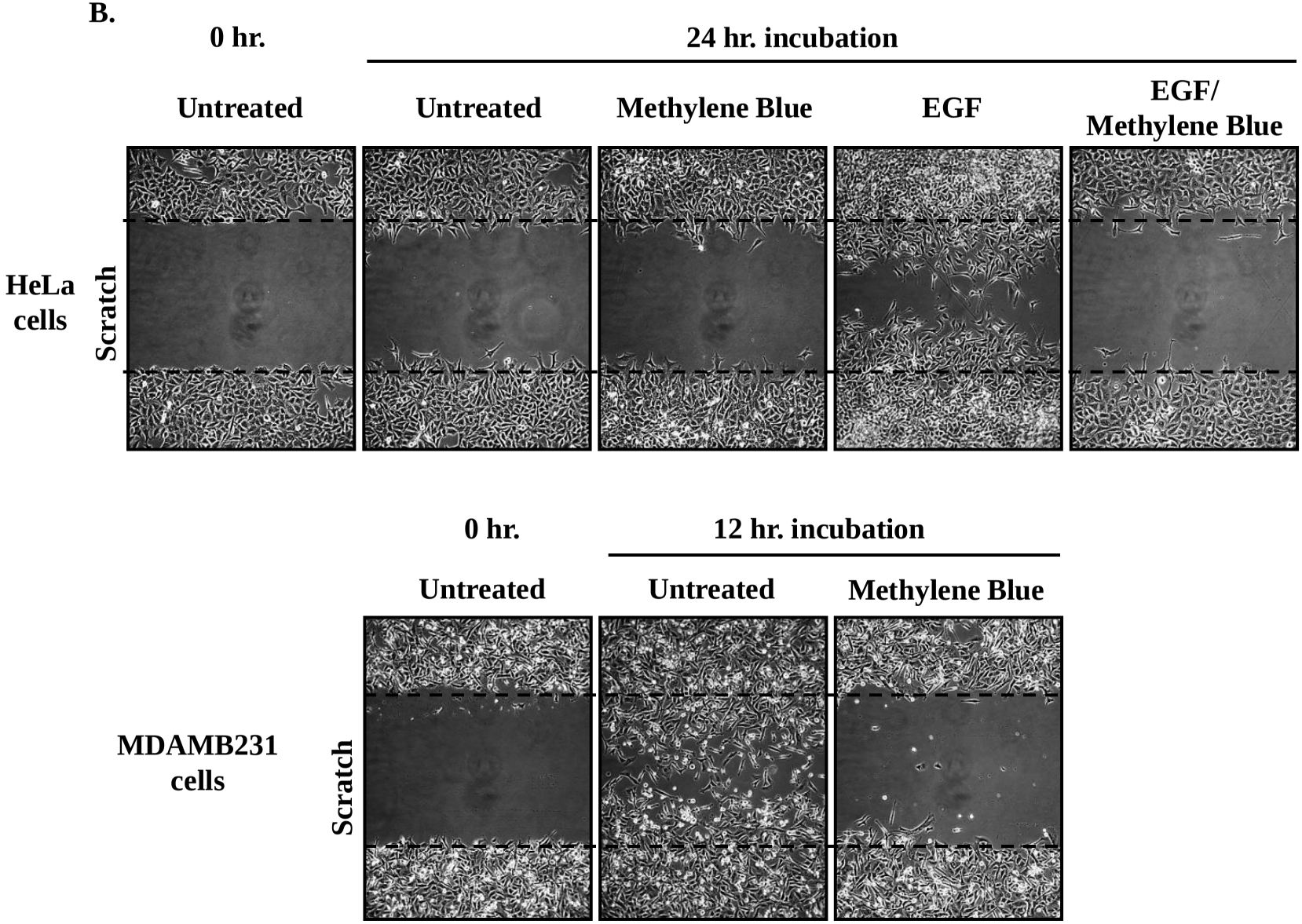
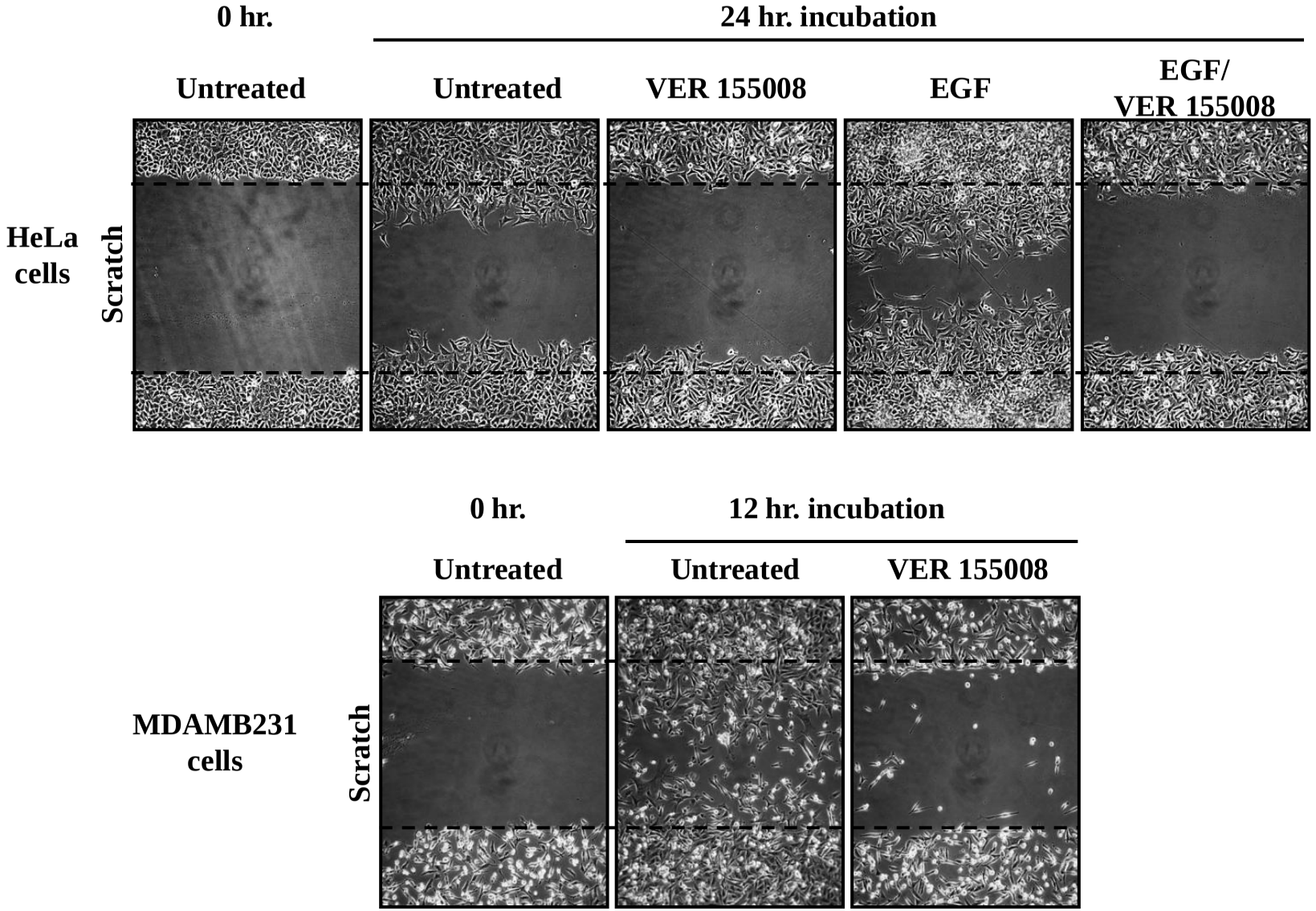


Figure 2.8 (Continued)

C.



invasive capabilities (1,3,5,7). For this reason, we have chosen to focus on the mechanisms used by cancer cells to enhance migration. We have previously identified tTG as a non-traditional signaling protein that is essential for the EGF-dependent migration of HeLa cells and the constitutive migration of MDAMB231 cells (11). Here we show that Hsp70 plays a critical regulatory role in the localization of tTG to the leading edges of actively migrating cells.

Given that EGF stimulation results in the accumulation of tTG at the leading edges of cells, our initial assumption was that a growth factor-induced trafficking event triggered the movement of a pool of cytosolic tTG to the plasma membrane. However, membrane fractionation studies revealed that a discrete population of tTG, which comprises ~10% of the tTG expressed in cells, constitutively associates with the plasma membrane, and is unaffected by EGF treatment. These findings supported the possibility that the membrane-associated pool of tTG is redistributed to leading edges following exposure to EGF. What is particularly interesting is that EGF can activate this membrane-bound pool of tTG on a similar time-scale as the localization of tTG to leading edges. This correlation leads us to suspect that the activation of the membrane-associated tTG, and its localization to the leading edge, are coupled and need to occur in a coordinated fashion to promote cell migration. Support for this idea comes from previous studies using HeLa cells where we found that ectopically expressed tTG showed protein crosslinking (transamidation) activity, but its ectopic expression alone was not sufficient for enhancing cell migration (11). We believe the reason for this is that the ectopically expressed tTG cannot localize to leading edges without EGF treatment and that such localization is essential for promoting cell migration.

Certainly an important question concerned how tTG is capable of associating with the plasma membrane so that it can ultimately localize to the leading edges of cells. While tTG

appears to have some intrinsic capability to associate with lipid bilayers, as indicated by liposome experiments, sequence and structure analyses do not provide obvious clues as to how this might occur. We also wondered how tTG is recruited in an EGF-dependent manner to the leading edge, and so we searched for interacting proteins that might help tTG to localize to these membrane sites. Immunoprecipitation of tTG from membrane extracts and subsequent mass spectrometry analysis of the immunocomplexes led to the identification of two heat shock protein 70 family members, Hsp70 and Hsc70, as novel binding partners for tTG. We further showed that Hsp70 and tTG co-localize to leading edges. This suggested that Hsp70 could indeed play a role in the ability of tTG to accumulate at these sites. Using several different inhibitors against the ATP hydrolytic activity of Hsp70, including myricetin, methylene blue, and VER 155008, we showed that blocking the chaperonin activity of Hsp70 prevented tTG and Hsp70 from co-localizing to leading edges. Importantly, we further demonstrated that the exclusion of tTG and Hsp70 from leading edges caused by inhibiting Hsp70 activity was specific for these two proteins. In the presence of these inhibitors, actin structures still formed at the leading edges of cells. Moreover, other leading edge-resident proteins including activated forms of the small GTPases Rac and Ras were still able to properly localize to these membrane sites even when the cells were treated with myricetin, thus indicating that this inhibitor is not having a global effect. Therefore, the chaperonin ability of Hsp70 appears to be critical for the localization of tTG to leading edges.

Surprisingly, treatment of cells with the Hsp70 inhibitors does not block the interaction of tTG and Hsp70, suggesting that tTG is not a client of Hsp70. However, given the effect of myricetin, methylene blue, or VER 155008 on the localization of tTG, these findings imply that the interaction of Hsp70 with a certain client protein(s) is necessary for regulating the

localization of tTG. It has been shown that a number of signaling proteins may be clients of heat shock proteins including Src, Raf, and Akt and that these chaperones may play an important role in extending their signaling lifetimes (30). This then raises some interesting questions for the future, such as whether specific clients of Hsp70 are required to help tTG properly localize to leading edges.

Previous work by us has shown that the transamidation activity of tTG is important for the process of cell migration, as inhibiting this activity with MDC blocks the EGF-dependent migration of HeLa cells, as well as the constitutive migration of MDAMB231 cells (11). However, this activity is not sufficient for enhancing cell migration in either case, thereby suggesting that in addition to becoming activated, tTG needs to localize to leading edges. Initially, we used a biochemical approach to define the region of tTG that was required for its association with membranes in hopes of designing a tTG mutant that retained protein crosslinking activity, but was defective for binding to the plasma membrane. Thus far, we have not been able to identify a point mutant that uncouples these two functions of tTG. However, our identification of Hsp70 as a novel binding partner of tTG, and the fact that these two proteins co-localize to leading edges with the localization being blocked by myricetin, as well as methylene blue and VER 155008, provided us with a nice system for specifically investigating the importance of the leading edge-localization of tTG. In particular, because these Hsp70 inhibitors do not affect the protein crosslinking activity of tTG, it is possible to uncouple this function from its leading edge localization and determine what effect this has on cell migration. Indeed, we have shown that by blocking either tTG protein crosslinking activity (using MDC), or its leading edge-localization (using myricetin, methylene blue, or VER 155008), we are able to inhibit cell migration, demonstrating that these two events are coupled.

These findings now raise a number of intriguing questions regarding the role of heat shock proteins in cancer progression. Recently, it was shown that Hsp70 and Hsc70 are uniquely capable of interacting with lipids, as they can insert into artificial lipid bilayers and selectively bind to phosphatidylserine (28). Others have demonstrated that Hsp70 can bind to cholesterol-rich microdomains in tumor cells (27). Still, the importance of the membrane association of Hsp70 and the consequences this holds for cancer progression, are not yet understood. Here we offer a possible insight into why these chaperones localize to the plasma membrane, namely, to regulate a key signaling molecule, tTG, and facilitate its redistribution to leading edges. We believe that the identification of Hsp70's client proteins will provide additional insights into the roles of Hsp70 and tTG in cell migration. All of this then leads to the question of why tTG needs to localize to leading edges. We believe that the coupling of tTG localization with the activation of its protein crosslinking activity should shed light on the identity of a key transamidation substrate for tTG. The recent development of "clickable" inhibitors against tTG, which bind to only the transamidation-active form of the enzyme, offer the possibility of visualizing tTG-catalyzed protein crosslinking activity in cells (33). These inhibitors may prove very useful for determining whether tTG is active specifically at leading edges. If this is the case, we will want to identify the substrate(s) of tTG located at these leading edges and determine how crosslinking contributes to enhanced cell migration.

Materials and Methods

Materials. All cell culture reagents, the Colloidal Blue staining kit, EGF, Lipofectamine, Lipofectamine 2000, protein G-beads, and V5 antibody, as well as the control, tTG, and Hsp70 siRNAs were from Invitrogen. Monodansylcadaverine (MDC), 6-diamidino-2-phenylindole

(DAPI), myricetin, methylene blue, and the fibronectin antibody were obtained from Sigma, while VER 155008 was from Tocris Biosciences. The tTG and actin antibodies were from Neomarkers, the IκBα, Hsp70, and cortactin antibodies were from Cell Signaling, and the HA and Myc antibodies were from Covance. The biotinylated pentylamine (BPA) was from Pierce, and T101 was from Zedira. The Quick Blue staining kit was from Boston Biologicals.

Cell culture. HeLa and MDAMB231 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS). The pcDNA3 constructs encoding V5-tagged Hsc70, the various forms of Myc-tagged tTG, and the HA-tagged forms of activated Rac and Ras, were transfected into cells using Lipofectamine, whereas the control, tTG, and Hsp70 siRNAs were introduced into cells using Lipofectamine 2000. As indicated, cell cultures or cell extracts were treated with various combinations of 0.1 μg/mL EGF, 50 μM myricetin, 10 μM methylene blue, 50 μM VER 155008, and 1.0 μM T101. The cells were then either collected for cell fractionation, fixed with 3.7% formaldehyde, or lysed with cell lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM $NaVO_4$, 1 mM β-glycerol phosphate, 1 μg/mL aprotinin, and 1 μg/mL leupeptin). The Bio-Rad DC protein assay was used to determine protein concentrations.

Immunofluorescence. The fixed cells were permeabilized with PBS containing 0.1% Triton X-100, blocked in PBS containing 10% bovine serum albumin (BSA), and incubated with the indicated primary antibodies for 2 hours. The cells were then incubated with either Oregon green 488- or Rhodamine red-conjugated secondary antibody (Molecular Probes) for 1 hour. Where indicated, Rhodamine-conjugated phalloidin was used to stain actin filaments, while

DAPI was used to stain nuclei. Following the secondary incubations, the cells were washed extensively with PBS, mounted, and visualized using the 63X objective on a Zeiss Axioskop fluorescent microscope. Images were captured and processed using IPLAB.

Cell migration (scratch) assays. Parental cells or cells expressing the control siRNA or tTG siRNAs were grown to confluence and then put in serum-free medium without or with 0.1 μg/mL EGF, and without or with 20 μM MDC, as indicated. Fifteen hours later, a wound was struck using a pipet tip and the culturing medium on the cells was replenished to remove detached cells. When examining the effects of myricetin, methylene blue, and VER 155008 on cell migration, cells maintained in serum-free medium for 15 hours, -/+ 0.1 μg/mL EGF, were treated without or with one of the inhibitors for one hour before striking a wound and replenishing the medium. After the indicated incubation period, the cells were fixed and visualized by light microscopy. Each of these experiments was performed at least 3 times.

Cell fractionation. To fractionate cells into their cytosolic and membrane components, harvested cells were re-suspended in homogenization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 200 mM sucrose, and 1 mM PMSF) and homogenized using a dounce homogenizer. The cell extracts were then centrifuged at 1,000 rpm for 10 minutes to remove the intact nuclei, followed by centrifugation at 47,000 rpm for 1.5 hours to pellet cellular membranes. The soluble cytosolic fraction was removed and saved, while the membrane fraction was lysed in membrane lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, and 0.5% Triton X-100) for 1 hour, followed by centrifugation at 13,000 rpm for 10 minutes to remove any insoluble material.

Transamidation assays. Cell lysates (15 μg of each) were incubated in a buffer containing 10 mM dithiothreitol, 10 mM $CaCl_2$, and 50 μM BPA for 10 minutes. The reactions were stopped with the addition of Laemmeli sample buffer, followed by boiling, and the proteins were resolved on a gel, transferred to polyvinylidene fluoride (PDVF) membranes, and blocked for at least 1 day in BBST (100 mM boric acid, 20 mM sodium borate, 0.01% SDS, 0.01% Tween 20, and 80 mM NaCl) containing 10% BSA. The membranes were incubated with horseradish-peroxidase-conjugated streptavidin, diluted at 1:2000 in BBST containing 5% BSA for 1 hour, and then washed extensively with BBST. The proteins that incorporated BPA were visualized on X-ray film after exposing the membranes to ECL reagent.

In vitro liposome fractionation assays. Synthetic liposomes were prepared from a lipid mixture containing 35% phosphatidylethanolamine, 25% phosphatidylserine, 5% phosphatidylinositol, and 35% cholesterol re-suspended in TBSM buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 2 mM $MgCl_2$). The lipids were extruded through an 8 micron filter, pelleted by centrifugation at 13,000 rpm for 15 minutes, and re-suspended in TBSM buffer. Equal amounts of the lipid preparation were then incubated with either recombinant wild-type tTG or BSA for 15 minutes, followed by centrifugation at 13,000 rpm for 10 minutes at room temperature. The supernatant was concentrated to ~30 μL using a microfuge concentrator with a 10K molecular weight cut-off, while the pelleted liposomes were re-suspended in 30 μL of TBSM buffer. Each of the samples was resolved on a gel and then stained with Quick Blue to detect proteins.

Immunoprecipitations. Cell lysates (~1.2 mg) that had been pre-cleared with protein G-beads were incubated with nothing, a Myc antibody or a non-specific mouse IgG control antibody for

1.5 hours as indicated. Protein G-beads were then added to the lysates and incubated for an additional 1.5 hours, at which time the beads were washed extensively with cell lysis buffer.

Immunoblot analysis. Whole cell extracts, various isolated subcellular fractions, as well as immunoprecipitated proteins were resolved by SDS-PAGE, followed by transfer to PVDF membranes. The membranes were incubated with the indicated primary antibodies diluted in TBST (in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20). The primary antibodies were detected with horseradish-peroxidase-conjugated secondary antibodies followed by exposure to ECL reagent. Some of the resulting blots were quantified using Image-J software.

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CHAPTER 3

Tissue Transglutaminase Promotes Cell Survival Through a Mechanism Involving c-Src and PI3-kinase[*]

Introduction

Tissue transglutaminase (tTG) functions as a GTPase and an acyl transferase that catalyzes the formation of protein crosslinks. tTG expression and activation are frequently up-regulated in human cancer, where it has been implicated in a number of aspects of cancer cell growth and metastasis, including cell survival and chemo-resistance. However, the extent to which tTG cooperates with oncogenic proteins within the context of a cancer cell, versus its intrinsic ability to confer transformed characteristics to cells, is poorly understood. To address this question, we asked what effect the ectopic expression of tTG in a normal (non-transformed) cellular background would have on the behavior of the cells. Using NIH3T3 fibroblasts stably expressing a Myc-tagged form of tTG, we found that tTG strongly protected these cells from serum-starvation-induced apoptosis and triggered the activation of the PI3-kinase/mTOR/p70 S6-kinase pathway. We determined that tTG forms a complex with the non-receptor tyrosine kinase c-Src and PI3-kinase, and that treating cells with inhibitors to block tTG function (monodansylcadaverine; MDC) or c-Src kinase activity (PP2) disrupted the formation of this complex, and prevented tTG from activating the PI3-kinase pathway. Moreover, treatment of fibroblasts over-expressing tTG with PP2, or with inhibitors that inactivate components of the PI3-kinase pathway, including PI3-kinase (LY294002) and mTOR (rapamycin), ablated the tTG-

[*] Submitted and under revision for publication as Boroughs LK & Antonayk MA, et al. (2013) *J Biol Chem.*

promoted survival of the cells. These findings demonstrate that tTG has an intrinsic capability to stimulate cell survival through a novel mechanism that activates PI3-kinase signaling events, thus highlighting tTG as a potential target for the treatment of human cancer.

Tissue transglutaminase (tTG) is a protein that is capable of multiple catalytic activities. In particular, tTG can bind and hydrolyze GTP like members of the large and small families of GTPases (i.e. Rho, Rac, Cdc42, and Ras) (1-3). It also exhibits a calcium-dependent acyl transferase activity (transamidation) that catalyzes the formation of an amide bond between the γ-carboximide group of a glutamine residue within one protein and the primary amino groups or the ε-amino group of a lysine residue within another protein (4,5). The generation of these protein-protein crosslinks by tTG has been implicated in the regulation of a wide array of cellular processes, ranging from the maintenance of the extracellular matrix and cell adhesion to the induction of cellular differentiation and apoptosis (6-10). However, tTG has also been suggested to play crucial roles in the progression of a number of human disease states. In particular, during the past decade, several laboratories, including our own, have shown that increases in tTG expression and/or its enzymatic transamidation activity are hallmarks of various types of human cancer including breast, brain, ovarian, and pancreatic cancer (11-16). In many of these same studies, it was also shown that knocking-down tTG expression by RNAi in cancer cell lines where it was aberrantly expressed, or interfering with its ability to crosslink proteins by treating the cells with inhibitors like MDC, either ablated the growth of the cancer cells or made them more sensitive to chemotherapy and other types of apoptotic-inducing cellular stress (11-13,16).

The indications that the over-expression of tTG contributes to tumor progression and metastasis raise an important question, namely to what extent are the contributions of tTG to cancer progression shaped by the cancer cell context and the various oncogenic signaling

proteins present within transformed cells, versus the intrinsic ability of tTG to alter normal cellular behavior. Indeed, various studies have suggested that tTG can work together with oncogenic signaling proteins in the background of a cancer cell (17-20). One example from studies performed in our laboratory involves the ability of tTG to influence the transformed characteristics of human breast cancer cells. In particular, we discovered, when using the human SKBR3 breast cancer cell line as a model, that tTG expression and activation were strongly up-regulated in an epidermal growth factor (EGF)-dependent manner. Moreover, tTG was essential for the EGF-stimulated growth of these cancer cells in monolayer, as well as for their anchorage-independent growth and importantly, their survival in the face of stress conditions and apoptotic challenges such as chemotherapeutic agents (20). We then demonstrated that a key element in the transformed characteristics of these breast cancer cells, as imparted by tTG, was its ability to form a complex with the non-receptor tyrosine kinase and proto-oncogene c-Src.

Here, we have set out to determine whether tTG has the ability to alter the behavior of normal (non-transformed) cells, as a means of obtaining insights into the capability of this protein in the absence of a cancer cell context to induce characteristics necessary for malignant transformation. To address this important question, we have examined the biological consequences of ectopically expressing tTG in NIH3T3 cells, a normal fibroblast cell line. Interestingly, we found that tTG strongly promoted NIH3T3 cell survival by enhancing the activation of the canonical PI3-kinase/mTOR/p70 S6-kinase pathway. We then went on to demonstrate that the ability of tTG to activate this signaling pathway was through the assembly of a complex consisting of tTG, c-Src, and PI3-kinase. Importantly, treating the cells with either the Src inhibitor, PP2, or the tTG inhibitor, MDC, disrupted the interaction between c-Src and tTG, as well as blocked the ability of tTG to stimulate PI3-kinase-mediated signaling events.

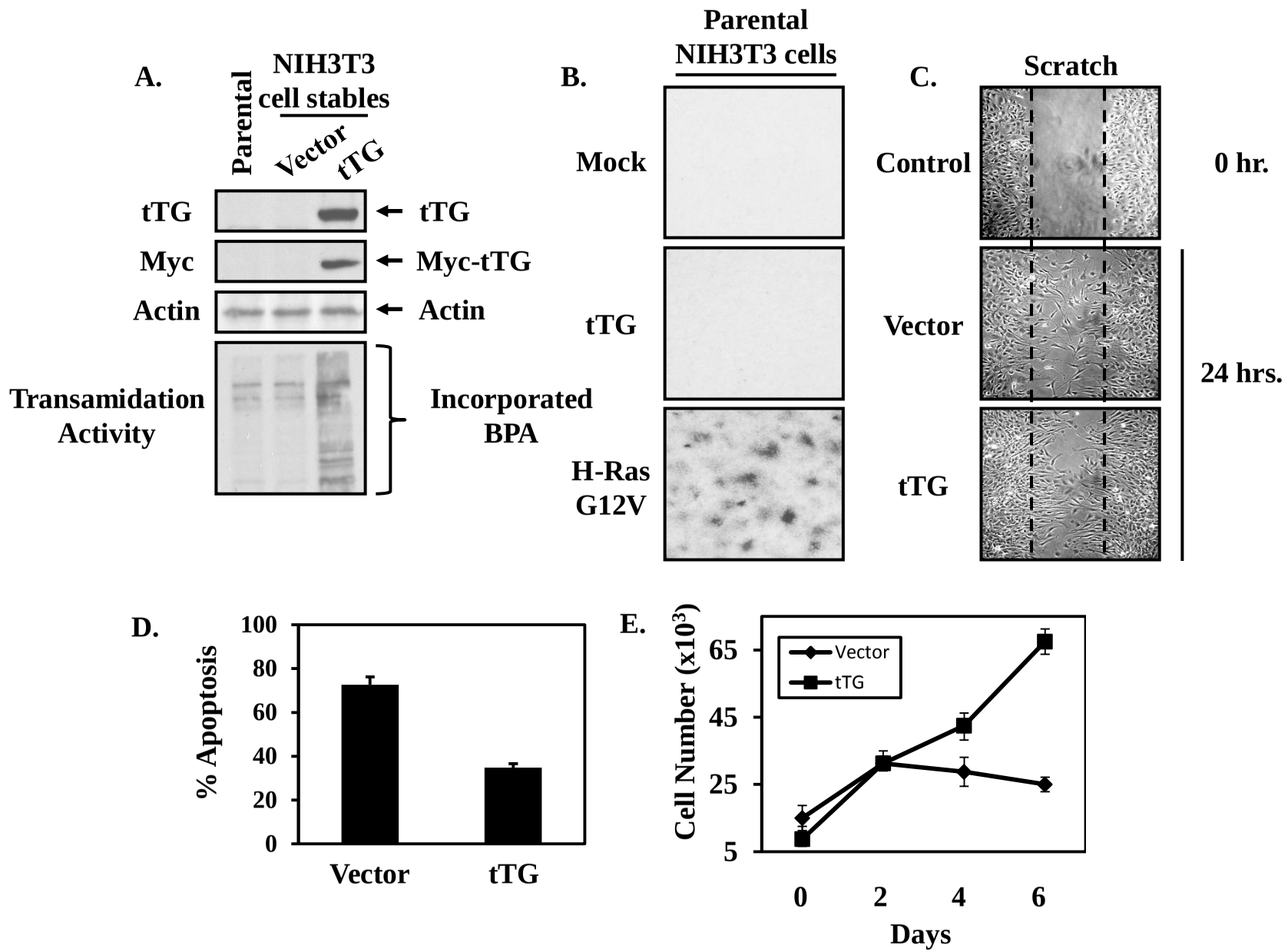
Thus, these findings point to tTG as being a key participant in a c-Src-PI3-kinase signaling pathway and that it is able to assemble this signaling complex even in a non-transformed cellular context. This capability is likely to have important consequences for enabling cancer cells to survive various apoptotic challenges including treatment with chemotherapeutic drugs.

Results

Ectopic expression of tTG in fibroblasts promotes cell survival

Increases in tTG expression and activation occur in several different types of human cancer resulting in a wide range of potential interactions and cross-talk with oncogenic proteins that drive transformation (12,14,20-22). This has contributed to some confusion in the field regarding the many possible roles that tTG plays in cancer progression. In particular, it has been extremely difficult to identify the inherent and fundamental actions of tTG that contribute to the development of the malignant state. In order to determine the intrinsic capability of tTG to mediate actions relevant to cancer progression, we examined the effects of over-expressing this protein in a normal (non-transformed) cell type. The NIH3T3 mouse fibroblast cell line was chosen as our model system for this study, as it is commonly used to read-out various types of cellular outcomes (23-25). Consistent with earlier findings, Figure 3.1A (*top panel, first lane*) shows that these cells fail to express detectable levels of tTG. We then generated two different NIH3T3 stable cell lines; one expressing the vector alone and the other expressing a Myc-tagged form of wild-type tTG (Figure 3.1A, *second panel* from the *top, second and third lanes*). The tTG ectopically expressed in the fibroblasts is functionally active, as indicated by its ability to catalyze the incorporation of biotinylated pentylamine (BPA) into lysate proteins, as compared to

Figure 3.1 Ectopic expression of tTG in NIH3T3 fibroblasts promotes cell growth and survival. (A) Whole cell lysates of parental NIH3T3 cells, or NIH3T3 cells stably expressing the vector alone or a Myc-tagged form of tTG, were immunoblotted with tTG, Myc, and actin antibodies. The same cell lysates were also assayed for transamidation (crosslinking) activity by determining the incorporation of BPA into lysate proteins as described in "Experimental Procedures". **(B)** Focus formation assays were carried-out on parental fibroblasts that were transiently transfected without (*Mock*), or with expression plasmids encoding either Myc-tagged tTG (*tTG*), or an HA-tagged activated form of Ras (*H-Ras G12V*). The cells were maintained in DMEM supplemented with 10% CS for 10 days, at which time they were fixed and stained with crystal violet. Shown are representative images of the resulting foci that formed for each condition. **(C)** Cell migration (scratch) assays were performed on NIH3T3 cells stably expressing the vector alone or a Myc-tagged form of tTG. Twenty-four hours after striking the wound, the cells were fixed and then visualized by light microscopy to determine the extent of wound closure. One set of vector alone-expressing fibroblasts was fixed immediately after striking the wound (*Control 0 hr.*) to indicate the width of the initial wound (indicated by *dashed lines*). **(D)** Cultures of the NIH3T3 cells stably expressing the vector alone or a Myc-tagged form of tTG were placed in serum-free medium for 36 hours, at which time they were collected and stained with DAPI to identify condensed and/or blebbed nuclei. Percent apoptosis was determined by calculating the ratio of apoptotic to non-apoptotic cells. The experiments were performed in triplicate and the results were averaged. The error bars indicate standard deviation. **(E)** Growth in low serum assays were performed on NIH3T3 cells stably expressing the vector alone or Myc-tagged tTG by plating them at a density of $2x10^4$ cells/dish in 6-well dishes and then placing them in DMEM containing 0.1% CS. Every other day for 6 days, one set of cells was counted, while on the remaining sets of cells the medium was replenished. The experiments were performed in triplicate and the results were averaged together and graphed. The error bars indicate standard deviation.


A.
NIH3T3 cell stables
Parental
Vector
tTG
tTG
Myc
Actin
tTG
Myc-tTG
Actin
Transamidation Activity
Incorporated BPA
B.
Parental NIH3T3 cells
Mock
tTG
H-Ras G12V
C.
Scratch
Control
Vector
tTG
0 hr.
24 hrs.
D.
% Apoptosis
100
80
60
40
20
0
Vector
tTG
E.
Cell Number (x10³)
65
45
25
5
Vector
tTG
0
2
4
6
Days

parental or vector alone-expressing fibroblasts, which have little if any detectable crosslinking activity (Figure 3.1A, *bottom panel*).

Previous work from our laboratory had suggested that over-expressing tTG in normal cell lines was not sufficient to induce transformation, as indicated by their inability to exhibit anchorage-independent growth (i.e. as assayed by colony formation in soft agar) (26). Here, we followed-up on these findings by performing focus formation assays on NIH3T3 cells ectopically expressing tTG. The ability of cells to form foci (i.e. distinct areas of high cell density) represents another indicator of cellular transformation that measures the ability of cells to overcome the contact inhibition exhibited by non-transformed cells when grown in monolayer. For these experiments, cultures of parental fibroblasts were transiently transfected without (*Mock*), or with expression plasmids encoding either tTG (*tTG*) or an oncogenic form of Ras (*H-Ras G12V*), and then were maintained in normal growth medium (DMEM containing 10% CS) for 10 days. The resulting cell cultures were then fixed and stained with crystal violet to highlight any differences in cell densities (i.e. foci) that might have occurred as an outcome of expressing tTG or activated H-Ras in the cells. As anticipated, fibroblasts expressing oncogenic Ras (*H-Ras G12V*) formed numerous foci (Figure 3.1B, *bottom panel*), while neither the control fibroblasts (*Mock*) (Figure 3.1B, *top panel*), nor the NIH3T3 cells transiently transfected with the tTG plasmid (Figure 3.1B, *middle panel*), showed detectable foci.

Given that tTG has been shown to localize to the leading edges of actively migrating cancer cells where it promotes the EGF-stimulated migration of the HeLa cervical carcinoma cell line, as well as the constitutive migration exhibited by the MDAMB231 breast cancer cell line (17,27), we next examined whether the over-expression of tTG in NIH3T3 cells would enhance their ability to migrate. The NIH3T3 cells stably expressing the vector alone or a Myc-tagged

form of tTG were subjected to wound healing (scratch) assays to determine whether there was any difference in the rate at which these cell lines migrated. Figure 3.1C shows that the extent of cell migration into the wound by fibroblasts expressing the vector alone, versus Myc-tagged tTG, was similar, suggesting that tTG is not important for promoting the general migration of these cells.

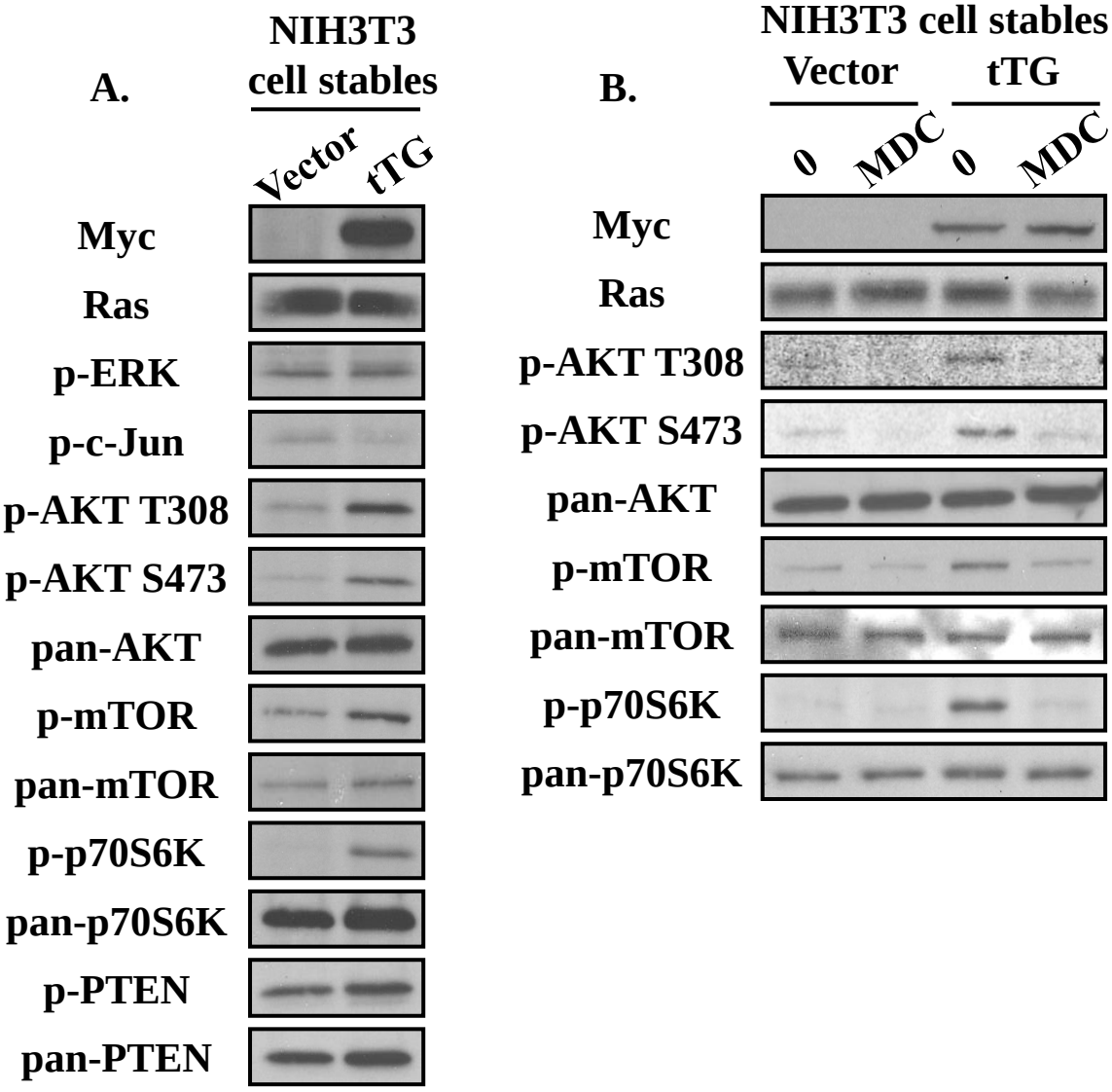
We then examined the ability of tTG to impact cell survival. Serum starvation is a stress that induces a cell death response in a number of cell types, including NIH3T3 fibroblasts (26,28). Thus, we took cultures of NIH3T3 cells stably expressing the vector alone or Myc-tagged tTG and placed them in serum-free medium for ~36 hours. The cells were then collected and the extent of cell death was determined by staining the cells with DAPI and examining them for the appearance of condensed and/or blebbed nuclei, an indicator of apoptosis. Figure 3.1D shows that ~70% of vector alone-expressing cells were apoptotic when cultured in medium lacking serum. In contrast, ~30% of the tTG-expressing fibroblasts were apoptotic under the same culturing conditions, suggesting tTG strongly promotes the survival of the fibroblasts. Similarly, the growth rates of the stable cell lines maintained in medium containing 0.1% serum (low serum conditions) over the course of 6 days were also assessed. The results in Figure 3.1E show that tTG promoted the growth of fibroblasts cultured under low serum conditions, as evidenced by the continued growth of the cells stably expressing tTG, whereas, the growth rate of the cells expressing the vector alone was stunted. This data suggests that tTG expression in a normal non-transformed cell type is sufficient to promote some aspects of cellular transformation, in particular, cell survival and growth in low serum.

tTG activates PI3-kinase

To further explore how tTG promotes cell survival, the NIH3T3 stable cell lines were maintained in serum-free medium for 24 hours and lysed. The whole cell lysates were then subjected to Western blot analysis using antibodies which detect the activated or phosphorylated forms of several traditional signaling proteins known to promote cell survival, with the expectation being that if tTG promotes cell viability by activating certain signaling proteins, then we should be able to detect an increase in the activation of these proteins in the fibroblasts expressing Myc-tagged tTG compared to the cells expressing the vector alone. The proteins whose activities were examined included members of the mitogen activated protein (MAP) kinase family, specifically, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), as well as components of the canonical PI3-kinase signaling cascade, namely, AKT, mammalian target of rapamycin (mTOR), and p70 S6-kinase (p70S6K). Figure 3.2A shows that when using antibodies recognizing the activated form of ERK, as well as the phosphorylation of the transcription factor c-Jun at Ser63 and Ser73 (i.e. two known JNK phosphorylation sites), we found that neither of these MAP kinase family members were activated by tTG over-expression (*third and fourth panels* from the *top*, respectively). However, components of the PI3-kinase pathway were significantly affected. Specifically, fibroblasts ectopically expressing tTG showed higher levels of AKT (*fifth and sixth panels* from the *top*), mTOR (*eighth panel* from the *top*), and p70 S6-kinase activity (*tenth panel* from the *top*) compared to control cells, while the overall expression levels of each of these signaling proteins remained constant (*seventh, ninth, and eleventh panels* from the *top*, respectively).

To further confirm that these results were due to the ectopic expression of tTG, we treated the stable cell lines without or with the tTG inhibitor, MDC, which competes with

Figure 3.2 tTG promotes activation of the PI3-kinase/mTOR/p70 S6-kinase pathway. (A) NIH3T3 cells stably expressing the vector alone or a Myc-tagged form of tTG were placed in serum-free medium for 24 hours, at which time they were lysed and subjected to Western blot analysis using the indicated antibodies. **(B)** The same stable cell lines were cultured in serum-free medium supplemented without (*0*) or with MDC for 24 hours at which time they were lysed. The whole cell lysates were then subjected to Western blot analysis using the indicated antibodies.


A.
NIH3T3 cell stables
Vector
tTG
Myc
Ras
p-ERK
p-c-Jun
p-AKT T308
p-AKT S473
pan-AKT
p-mTOR
pan-mTOR
p-p70S6K
pan-p70S6K
p-PTEN
pan-PTEN
B.
NIH3T3 cell stables
Vector
tTG
0
MDC
0
MDC
Myc
Ras
p-AKT T308
p-AKT S473
pan-AKT
p-mTOR
pan-mTOR
p-p70S6K
pan-p70S6K

substrates for binding to the transamidation active site. Figure 3.2B shows that MDC treatment blocked the increases in AKT, mTOR, and p70 S6-kinase activities observed in fibroblasts over-expressing tTG (*third, fourth, sixth, and eighth panels* from the *top*, respectively), again without altering the expression levels of each of these proteins (*fifth, seventh, and ninth panels* from the *top*, respectively).

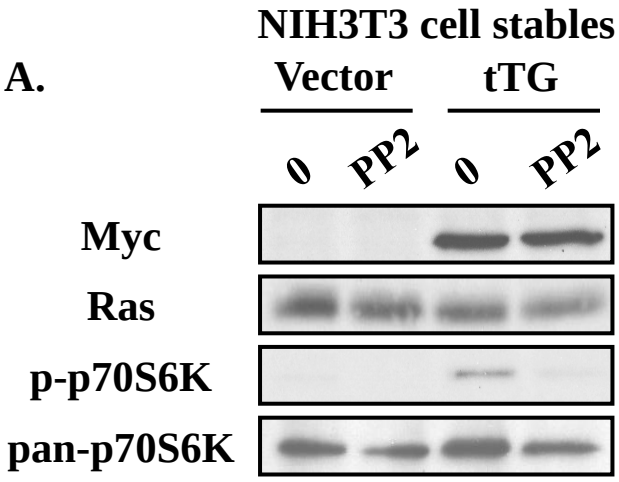
The ability of tTG to activate the PI3-kinase pathway requires Src kinase activity

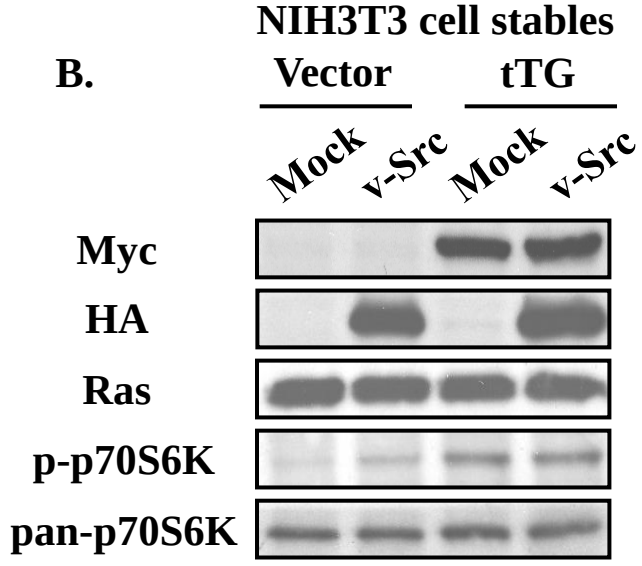
We wanted to learn more about how the over-expression of tTG in NIH3T3 cells leads to increased activation of PI3-kinase and several of its downstream effectors, namely AKT, mTOR, and p70 S6-kinase. Based on a recent suggestion that tTG can down-regulate the expression of the lipid phosphatase and major negative regulator of PI3-kinase signaling, PTEN (for phosphatase and tensin homologue deleted on chromosome ten), in pancreatic cancer cells (22), we first examined whether tTG was similarly affecting PTEN expression to promote PI3-kinase signaling activity in NIH3T3 cells. However, this was ruled out when we compared the levels of PTEN in the lysates collected from the NIH3T3 fibroblasts expressing the vector alone to those expressing Myc-tagged tTG and found that they were similar (Figure 3.2A, *bottom panel*). Moreover, we also determined whether the phosphorylation of PTEN was increased in the fibroblasts over-expressing tTG, as the phosphorylation of PTEN in its C-terminal tail has been shown to inhibit its phosphatase activity, as well as enhance its stability (29,30). However, the relative amounts of phosphorylated PTEN detected in these same whole cell lysates were nearly identical (Figure 3.2A, *second panel* from the *bottom*), further confirming that tTG is not stimulating the PI3-kinase pathway by inhibiting PTEN.

The non-receptor tyrosine kinase c-Src, as well as other members of the Src family, can bind and phosphorylate the p85 regulatory subunit of PI3-kinase (31-33). This phosphorylation event is believed to induce a conformational change in p85 that allows the p110 catalytic subunit of PI3-kinase to become activated and signal to its downstream effectors (i.e. AKT, mTOR, and p70 S6-kinase) (34,35). Moreover, it has been shown that the ability of c-Src to stimulate PI3-kinase activity is critical for Src-mediated cellular transformation, highlighting that these two proteins can participate in a common signaling pathway important for malignant transformation (36,37). Interestingly, our laboratory recently reported that tTG can bind and activate c-Src in human SKBR3 breast cancer cells stimulated with EGF (20). Since c-Src interacts with both PI3-kinase and tTG, and the over-expression of tTG in fibroblasts enhances the activation of the PI3-kinase pathway, we asked whether Src activity was important for the ability of tTG to stimulate PI3-kinase/mTOR/p70 S6-kinase signaling. To address this question, serum-starved fibroblasts stably expressing the vector alone or Myc-tagged tTG were incubated without or with the Src kinase inhibitor PP2, before being lysed and then immunoblotted to read-out the effects of this treatment on p70 S6-kinase activity. Figure 3.3A shows that inhibiting Src in cells expressing Myc-tagged tTG reduced the levels of p70 S6-kinase activity (*third panel* from the *top*) to those observed in control cells expressing the vector alone. Thus, c-Src kinase activity is indeed necessary for tTG to activate the PI3-kinase pathway.

This finding prompted us to then determine whether the activation of c-Src was sufficient to mimic the effects of tTG and stimulate PI3-kinase-mediated signaling events. NIH3T3 cells stably expressing the vector alone or a Myc-tagged form of tTG were transiently transfected without (*Mock*) or with an HA-tagged mutant form of Src that lacks a critical C-terminal inhibitory phosphorylation site, making it constitutively active (HA-*v-Src*) (38). The

Figure 3.3 Src activity is required for tTG-stimulated PI3-kinase activation. (A) NIH3T3 cells stably expressing the vector alone or Myc-tagged tTG were placed in serum-free medium for 12 hours, at which time they were treated without (*0*) or with PP2 for an additional 12 hours. The cells were then lysed and the cell extracts were subjected to Western blot analysis using the indicated antibodies. **(B)** The stable cell lines were transfected without (*Mock*) or with an HA-tagged constitutively active form of Src (*v-Src*) and then were maintained in serum-free medium for 12 hours. The cells were lysed and the extracts subjected to Western blot analysis using the indicated antibodies.


A.
NIH3T3 cell stables
Vector
tTG
0
PP2
0
PP2
Myc
Ras
p-p70S6K
pan-p70S6K


B.
NIH3T3 cell stables
Vector
tTG
Mock
v-Src
Mock
v-Src
Myc
HA
Ras
p-p70S6K
pan-p70S6K

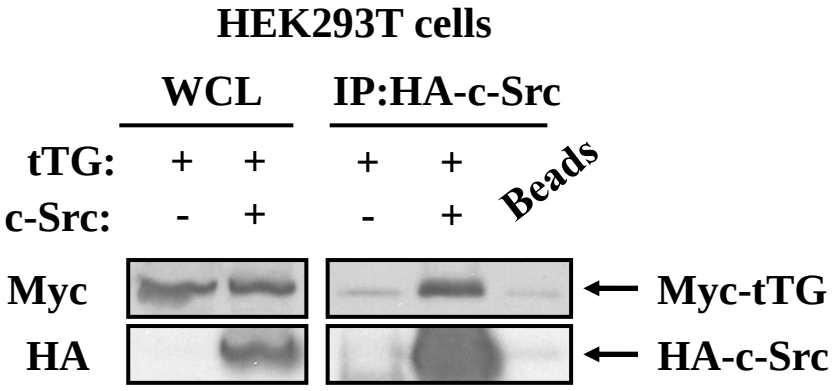
transfectants were maintained in serum-free medium for 12 hours, before being lysed and subjected to Western blot analysis. Using p70 S6-kinase activation as the read-out for activation of the PI3-kinase pathway, we found that despite the relatively high expression of HA-tagged v-Src in the vector alone-expressing cells (Figure 3.3B, *second panel* from the *top*), only a modest increase in p70 S6-kinase activity (*fourth panel* from the *top*) was detected, that was significantly less than the activity observed in fibroblasts over-expressing tTG. It is also worth noting that the ectopic expression of HA-tagged v-Src in cells stably expressing tTG did not enhance p70 S6-kinase activity beyond that observed in cells stably expressing tTG alone. Taken together, these results suggest that while c-Src kinase activity is necessary for tTG to activate PI3-kinase, it is not sufficient to completely mimic the effects of tTG.

tTG forms a complex with Src and PI3-kinase

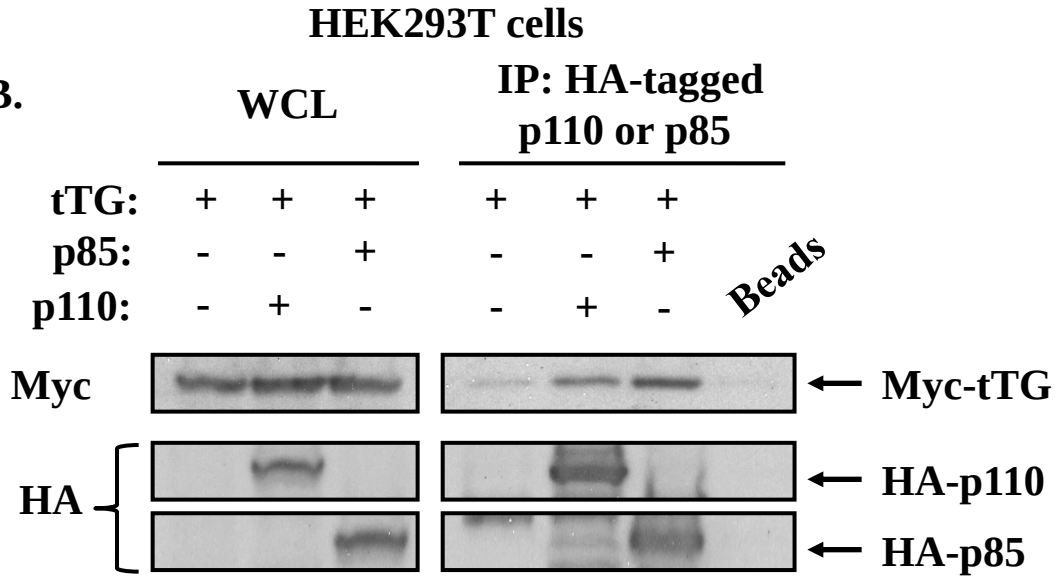
How then does tTG cooperate with c-Src to activate the PI3-kinase pathway? Since Src was previously shown to bind and phosphorylate the p85 regulatory subunit of PI3-kinase, which induces the activation of its p110 catalytic subunit, we examined the possibility that tTG stimulates PI3-kinase activity by enhancing the ability of Src to interact with and phosphorylate p85. Whole cell lysates generated from HEK293T cells that had been transiently transfected with a Myc-tagged tTG construct, alone, or together with an HA-tagged c-Src construct (Figure 3.4A, *left panels*) were subjected to immunoprecipitations using an HA antibody. Figure 3.4A (*right panels*) shows that, indeed, tTG co-immunoprecipitates with c-Src, similar to what our laboratory has shown in the past (20). Surprisingly, we also found that tTG is capable of interacting with PI3-kinase. For these experiments, rather than transiently expressing Myc-tagged tTG together with HA-tagged c-Src, we instead co-expressed Myc-tagged tTG together

Figure 3.4 tTG binds Src and PI3-kinase. (A) Immunoprecipitations were performed using an HA antibody on whole cell lysates collected from HEK293T cells ectopically expressing Myc-tagged tTG, or Myc-tagged tTG and HA-tagged c-Src. A beads only control was included to confirm the specificity of the interaction. The whole cell lysates (*WCL*) and the resulting immunocomplexes (*IP:HA-c-Src*) were blotted with Myc and HA antibodies. **(B)** Immunoprecipitations were performed using an HA antibody on extracts collected from HEK293T cells ectopically expressing Myc-tagged tTG, or Myc-tagged tTG together with either the HA-tagged form of the p110 catalytic subunit of PI3-kinase (HA-p110) or the p85 regulatory subunit of PI3-kinase (HA-p85). A beads only control was included to confirm the specificity of the interactions. The whole cell lysates (*WCL*) and the resulting immunocomplexes (*IP:HA-tagged p110 or p85*) were blotted with Myc and HA antibodies.

A.


HEK293T cells
WCL
IP:HA-c-Src
tTG: + + + +
c-Src: - + - +
Beads
Myc
HA
Myc-tTG
HA-c-Src

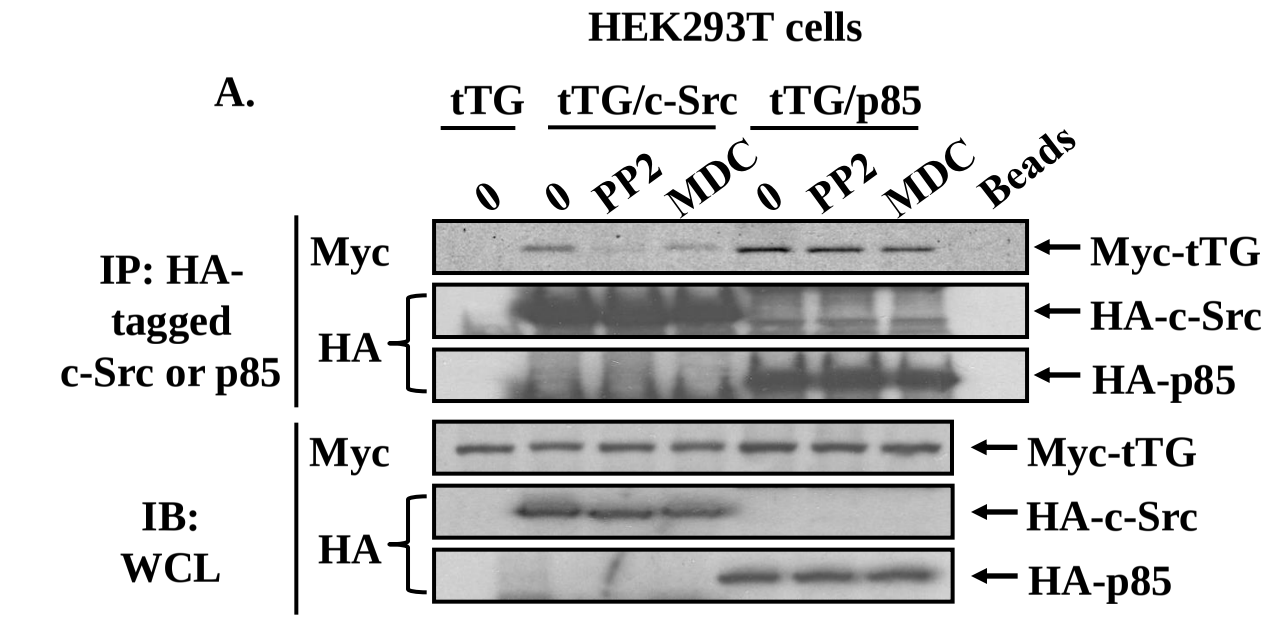
B.

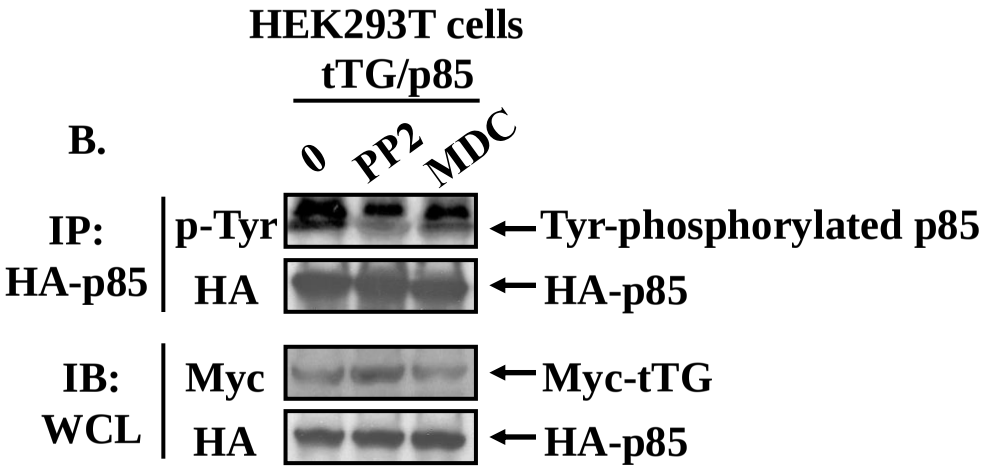

HEK293T cells
WCL
IP: HA-tagged p110 or p85
tTG: + + + + + +
p85: - - + - - +
p110: - + - - + -
Beads
Myc
HA
Myc-tTG
HA-p110
HA-p85

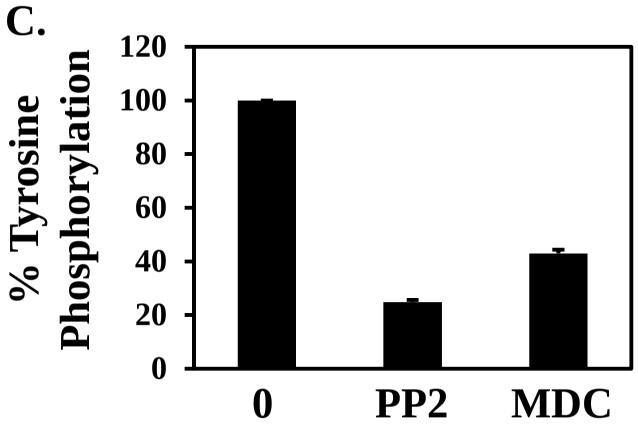
with one of the two subunits of PI3-kinase; the p110 catalytic subunit (HA-tagged p110) or the p85 regulatory subunit (HA-tagged p85). Figure 3.4B (*left panels*) shows that the ectopic expression of these constructs in cells was similar. Immunoprecipitations using an HA antibody performed on the whole cell lysates prepared from these transfectants showed that tTG co-immunoprecipitates with both of the PI3-kinase subunits (Figure 3.4B, *right panels*).

Is the formation of a complex between tTG, Src, and PI3-kinase responsible for the enhanced PI3-kinase signaling observed in the tTG-expressing fibroblasts? Given that the tTG inhibitor MDC and the Src inhibitor PP2 can abrogate the enhanced PI3-kinase signaling observed in fibroblasts stably expressing tTG, we reasoned that if the formation of a trimeric complex was important for this outcome, MDC and PP2 might then inhibit its formation. To test this, tTG was expressed alone, or together with c-Src or p85, in HEK293T cells and then the cells were treated without or with either PP2 or MDC for 6 hours prior to being lysed. The cell extracts were then incubated with an HA antibody to immunoprecipitate either the HA-tagged forms of c-Src or p85 expressed in the cell lysates (Figure 3.5A, *second and third panels* from the *top,* respectively). The resulting immunocomplexes were also immunoblotted with a Myc antibody to detect the extent that tTG interacted with c-Src or p85 under conditions where Src or tTG activity was inhibited. Figure 3.5A (*top panel*) shows that the interaction between tTG and p85 was insensitive to treatment with either PP2 or MDC, suggesting that the ability of these to proteins to interact with one another does not require c-Src kinase activity, nor access to the transamidation active site of tTG. On the other hand, PP2 and MDC blocked the ability of tTG to co-immunoprecipitate with Src, suggesting that the activity of both of these proteins promotes the ability of Src to interact with tTG.

Figure 3.5 Inhibiting tTG or Src activity blocks the ability of tTG to bind Src and the ability of Src to phosphorylate p85. **(A)** HEK293T cells ectopically expressing Myc-tagged tTG (*tTG*), Myc-tagged tTG and HA-tagged c-Src (*tTG/c-Src*), or Myc-tagged tTG and the HA-tagged p85 regulatory subunit of PI3-kinase (*tTG/p85*), were treated without (*0*) or with PP2 or MDC for 6 hours and then lysed. The cell lysates were subjected to immunoprecipitations using an HA antibody. A beads only control was included to confirm the specificity of the interactions. The whole cell lysates (*WCL*) and the resulting immunocomplexes (*IP:HA-tagged c-Src or p85*) were blotted using Myc and HA antibodies. **(B)** HEK293T cells co-expressing Myc-tagged tTG and HA-tagged p85 (*tTG/p85*) were treated without (*0*) or with PP2 or MDC for 6 hours and then lysed. The lysates were subjected to immunoprecipitations using an HA antibody. The whole cell lysates (*WCL*) were blotted with Myc and HA antibodies. The resulting immunocomplexes (*IP:HA-p85*) were first blotted with a phosphotyrosine antibody. The blot was then stripped and re-probed with an HA antibody to confirm that an equal amount of p85 was immunoprecipitated for each condition. **(C)** The experiment shown in "B" was performed in triplicate and the extent of HA-tagged p85 tyrosine phosphorylation detected by Western blot analysis was quantified for each condition and averaged and graphed. The error bars represent standard deviation.


HEK293T cells
A.
tTG
tTG/c-Src
tTG/p85
0
0
PP2
MDC
0
PP2
MDC
Beads
IP: HA-tagged c-Src or p85
Myc
HA
Myc-tTG
HA-c-Src
HA-p85
IB: WCL
Myc
HA
Myc-tTG
HA-c-Src
HA-p85


HEK293T cells
tTG/p85
B.
0
PP2
MDC
IP: HA-p85
p-Tyr
HA
Tyr-phosphorylated p85
HA-p85
IB: WCL
Myc
HA
Myc-tTG
HA-p85


C.
% Tyrosine Phosphorylation
120
100
80
60
40
20
0
0
PP2
MDC

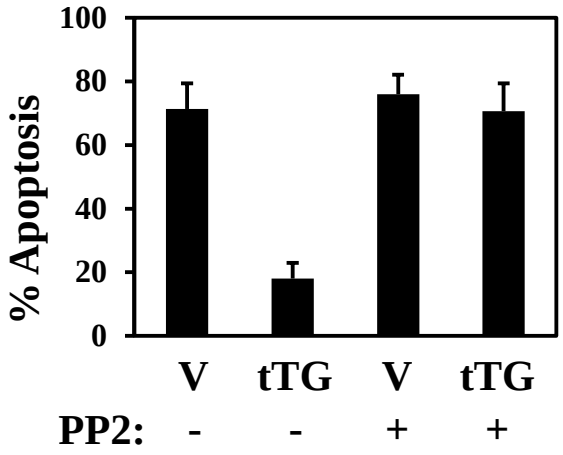
Src has been shown to phosphorylate p85 and to stimulate the signaling capability of PI3-kinase (31). This, coupled with the fact that we find tTG is able to form a complex with both PI3-kinase and c-Src, raised the interesting possibility that tTG functions as a scaffold to more efficiently bring Src and PI3-kinase together. To test this, Myc-tagged tTG was transiently expressed along with HA-tagged p85 in HEK293T cells, and then the cells were incubated without or with PP2 or MDC. The cells were lysed and the HA-tagged p85 was immunoprecipitated from the extracts using an HA antibody. The resulting immunocomplexes were first immunoblotted with a phosphotyrosine antibody to detect whether p85 was phosphorylated. Figure 3.5B (*top panel*) shows that p85 is indeed phosphorylated in the control lysates (*0*), whereas the tyrosine phosphorylation is decreased by ~80% with PP2 treatment and ~60% with MDC. The blot was then stripped and re-probed with HA antibody to confirm that an equivalent amount of p85 was immunoprecipitated for each condition (Figure 3.5B, *second panel* from the *top*). These experiments were performed multiple times and the results from each experiment were quantified and graphed (Figure 3.5C).

Inhibiting Src and components of the PI3-kinase pathway eliminate tTG-enhanced cell survival

The over-expression of tTG in NIH3T3 cells protects them from serum-starvation-induced apoptosis, as well as increases the activities of AKT, mTOR, and p70 S6-kinase, leading us to propose that tTG-promoted cell survival is dependent on its ability to activate the PI3-kinase pathway. Thus, we examined whether treating serum-starved fibroblasts expressing tTG with inhibitors of different components of this pathway, including PP2 (to inhibit Src activity), LY294002 (to inhibit PI3-kinase activity), and rapamycin (to inhibit mTOR activity), blocked the protective effect of tTG and re-sensitized the cells to serum-starvation-induced apoptosis.

Figure 3.6 Inhibition of Src, PI3-kinase, or mTOR blocks the tTG-enhanced cell survival. (A and B) NIH3T3 cells stably expressing the vector alone or Myc-tagged tTG were placed in serum-free medium supplemented without or with PP2, LY294002, or rapamycin for 36 hours, at which time the cells were collected and stained with DAPI to identify condensed and/or blebbed nuceli. Percent apoptosis was determined by calculating the ratio of apoptotic to non-apoptotic cells. The experiments were performed in triplicate and the results were averaged. The error bars indicate standard deviation.

A.


100
80
60
40
20
0
% Apoptosis
V
tTG
V
tTG
PP2:
-
-
+
+

B.

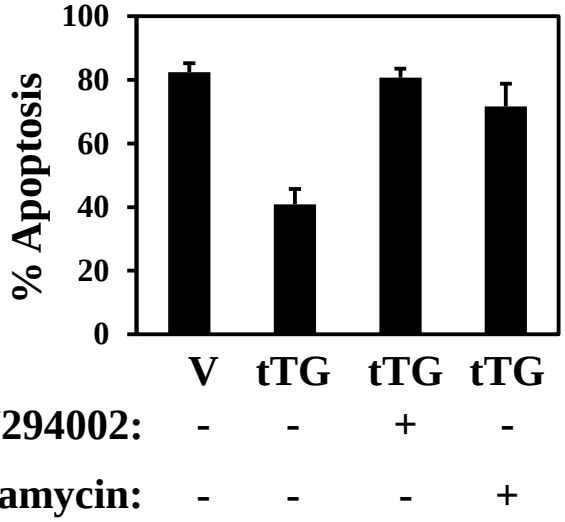

100
80
60
40
20
0
% Apoptosis
V
tTG
tTG
tTG
LY294002:
-
-
+
-
Rapamycin:
-
-
-
+

Figure 3.6A shows again that tTG over-expression strongly prevented the cells deprived of serum from undergoing cell death. However, the protective effect of tTG was completely ablated when the cells were treated with PP2. Likewise, LY294002 and rapamycin treatment blocked the ability of tTG to promote cell survival (Figure 3.6B), suggesting that tTG-promoted survival depends on its ability to activate the PI3-kinase signaling pathway. This led us to propose the model shown in Figure 3.7, where tTG, functioning as a scaffold, brings c-Src and PI3-kinase in proximity to one another. Src is then able to phosphorylate the p85 regulatory subunit of PI3-kinase, leading to the activation of the p110 catalytic subunit and its downstream effectors, AKT, mTOR, and p70 S6-kinase, which ultimately results in enhanced cell survival.

Discussion

There have been an increasing number of studies implicating tTG in cancer progression. tTG expression is frequently up-regulated in a number of types of human cancer, particularly those that are highly aggressive, metastatic, and chemo-resistant (11-16,21,39). Importantly, several of these studies have shown that knocking-down tTG expression in cancer cells with siRNAs, or treating cells with competitive inhibitors that bind at the transamidation active site of tTG, often sensitizes these cancer cells to chemotherapeutic agents and reduces many of their transformed features (11-16). While these findings point to tTG as being an important player in cancer progression, exactly how tTG contributes to the malignant state and whether it represents a valid target for cancer therapy, remains to be determined.

In this study, our goal was to use a simplified model system to better understand the intrinsic capability of tTG to confer cellular changes that might shed important light on the role that it plays in cancer progression. Thus, we sought to examine the actions of tTG in a non-

Figure 3.7 Model depicting how tTG brings Src and PI3-kinase together to enhance signaling and promote cell survival. Our results suggest that tTG functions as a scaffold and brings Src and PI3-kinase into close proximity to one another. Src is then able to phosphorylate the p85 regulatory subunit of PI3-kinase causing it to adopt a conformation that allows the p110 catalytic subunit of PI3-kinase to become activated and signal to its downstream effectors. The tTG-mediated activation of the PI3-kinase pathway promotes cell survival.

p110
p85
Src
tTG
tTG
p110
p85
Src
P
Enhanced signaling to AKT/mTOR/p70 S6-kinase
Enhanced cell survival

transformed cellular context, specifically NIH3T3 fibroblasts, so as to avoid the aberrant oncogenic signaling inputs characteristic of transformed/cancer cells that might complicate the picture. In fact, we found that the stable over-expression of tTG in NIH3T3 fibroblasts did not cause the cells to lose their normal contact inhibition and form foci when grown in monolayer, nor did it enhance their migration. However, interestingly, we discovered that tTG provided a marked survival advantage to the cells, when challenged with serum starvation. This finding was consistent with our earlier observations that tTG confers cancer cells with a strong protective effect against apoptotic stresses, such as those elicited by treatment of cells with chemotherapeutic agents, and thus potentially would explain the advantage provided by the over-expression of tTG in high grade and highly aggressive forms of human cancer (6,39)

So how does the over-expression of tTG in non-transformed fibroblasts enhance their survival capability? We obtained an interesting clue when we analyzed the activities of components of the MAP kinase and PI3-kinase signaling pathways and found that significant increases in the activation of AKT, mTOR, and p70 S6-kinase occurred in the tTG-expressing fibroblasts, compared to those expressing the vector alone. These results led us to suspect that tTG promotes cell survival by enhancing the activation of the PI3-kinase/mTOR/p70 S6-kinase pathway.

This raised an important question, namely, how is tTG able to activate the PI3-kinase pathway? While there had been suggestions in the literature that tTG may regulate the levels of PTEN, and in turn, enhance AKT activation (22), we assessed the levels of PTEN in the NIH3T3 stable cell lines and found that they were unaffected by tTG. This led us to examine whether c-Src is contributing to the activation of PI3-kinase in tTG-expressing fibroblasts. c-Src binds PI3-kinase and requires this interaction to promote cellular transformation (31-33,36,37). Given our

recent findings in the human breast cancer cell line SKBR3, that tTG is able to bind and increase c-Src activity in response to EGF treatment (20), we wondered whether a similar mechanism might be operating in fibroblasts over-expressing tTG. Indeed, we found that the activation of the PI3-kinase pathway to mTOR, and p70 S6-kinase in NIH3T3 cells over-expressing tTG is sensitive to Src inhibition.

Since we had shown previously that Src co-immunoprecipitates with tTG, and it has been reported that Src can bind to and activate PI3-kinase, we examined the possibility that Src, tTG, and PI3-kinase formed a 'signaling complex'. Indeed, we discovered that in addition to binding Src, tTG can be co-immunoprecipitated with both the p110 catalytic subunit and the p85 regulatory subunit of PI3-kinase. We reasoned that if the assembly of a complex that includes tTG, Src, and PI3-kinase is essential for sending a survival signal, then we would expect that inhibitors like MDC and PP2 which block these signals would have a corresponding inhibitory effect on complex formation. Interestingly, while inhibiting tTG and Src activity had no effect on the ability of tTG to be co-immunoprecipitated with p85, we found that disabling these signaling proteins completely blocked the interaction between tTG and Src. These results suggest that the tTG-p85 interaction is not dependent on having access to the transamidation active site of tTG, which would be blocked by MDC, whereas, the ability of Src to bind tTG requires such access. This observation correlates with our previous finding in breast cancer cells that Src is unable to bind a transamidation-defective form of tTG generated by mutation of the active site cysteine, C277V (20). These results imply that Src and p85 have different requirements for binding to tTG and do so in separate regions of tTG. Importantly, however, disrupting the ability of tTG to associate with Src eliminates the Src-mediated tyrosine

phosphorylation of p85, which in turn reduces the activation of the PI3-kinase signaling pathway.

Collectively, these findings point to a novel mechanism by which tTG is capable of triggering signals essential for cell survival (Figure 3.7). Given the established link between Src and PI3-kinase, it is clear that these two proteins can work together to promote cellular transformation, particularly in cells expressing oncogenic Src mutants. However, we now find that tTG, when over-expressed even in a non-transformed cellular setting, is capable of facilitating the assembly of a complex that includes c-Src and PI3-kinase, apparently bringing them into close proximity so as to allow Src to directly phosphorylate PI3-kinase, resulting in its activation and an ensuing stimulation of cell survival. These findings also offer important insights into how the over-expression of tTG in cancer cells can have important consequences that enable their survival in the face of stress and apoptotic signals such as chemotherapeutic drugs, and thus highlight tTG as a potentially an important therapeutic target in human cancer.

Materials and Methods

Materials. All cell culture reagents (unless mentioned otherwise), Lipofectamine, and protein G agarose beads were from Invitrogen. Monodansylcadaverine (MDC) and 6-diamidino-2-phenylindole (DAPI) were obtained from Sigma, while PP2, LY294002, and rapamycin were from Calbiochem. Biotinylated pentylamine (BPA) was obtained from Pierce, and the Myc and HA antibodies were from Covance. The Ras and pan-p70 S6-kinase antibodies were from Millipore, the tTG antibody was from Zedira, and the actin antibody was from Neomarkers. The anti-phosphotyrosine antibody, as well as the antibodies that recognize the total, activated, and/or

phosphorylated forms of ERK, c-Jun, AKT, mTOR, p70 S6-kinase, and PTEN were from Cell Signaling.

Cell Culture. Parental NIH3T3 cells were grown in DMEM containing 10% calf serum (CS), while HEK293T cells were grown in DMEM containing 10% fetal bovine serum (FBS). The pcDNA3 constructs encoding the Myc-tagged form of tTG and the HA-tagged forms of H-Ras G12V, c-Src, v-Src, p85, or p110, were transfected into cells using Lipofectamine. Clones of NIH3T3 mouse fibroblasts stably expressing the vector alone or a Myc-tagged form of wild-type tTG were selected by culturing the cells in DMEM containing 10% CS and 2 μg/mL puromycin. Once individual clones expressing either the vector alone or the Myc-tagged form of tTG were obtained, the cells were then maintained in the same growth medium supplemented with 0.5 μg/mL puromycin. Where indicated, cells were treated with 50 μM MDC, 10 μM PP2, 10 μM LY294002, or 50 nM rapamycin. Cells were lysed with cell lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM $NaVO_4$, 1 mM β-glycerol phosphate, 1 μg/mL aprotinin, 1 μg/mL leupeptin). The Bio-Rad DC protein assay was used to determine the protein concentrations of the cell lysates.

Transamidation Activity Assays. Fifteen micrograms of whole cell extracts collected from parental NIH3T3 cells or NIH3T3 cells stably expressing the vector alone or a Myc-tagged form of tTG, were incubated in a buffer containing 10 mM dithiothreitol, 10 mM $CaCl_2$, and 50 μM BPA for 10 minutes followed by the addition of Laemmli sample buffer. The samples were boiled, subjected to SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes, and blocked for one day in BBST (100 mM boric acid, 20 mM sodium borate, 0.01% SDS, 0.01%

Tween 20, 80 mM NaCl) containing 10% bovine serum albumin (BSA). The PVDF membranes were then incubated with horseradish-peroxidase-conjugated streptavidin at a dilution of 1:2000 in BBST containing 5% BSA for one hour, followed by extensive washing with BBST. The membranes were then exposed to ECL reagent and the proteins that had incorporated BPA were visualized using x-ray film.

Focus Formation Assays. Nearly confluent cultures of fibroblasts were transfected without (Mock) or with expression plasmids encoding a Myc-tagged form of tTG or an HA-tagged form of activated H-Ras (H-Ras G12V) and were maintained in DMEM containing 10% CS for 10 days. The cells were then fixed with 3.7% formaldehyde and stained with 0.4% crystal violet to visualize any resulting foci that formed.

Cell Migration (Scratch) Assays. Multiple sets of NIH3T3 cells stably expressing either the vector alone or a Myc-tagged form of tTG were grown to confluence, at which time a wound was struck down the center of each plate using a pipet tip and the cultures rinsed with phosphate buffered saline to remove the detached cells. One set of cells was immediately fixed with 3.7% formaldehyde after striking the wound to indicate the size of the initial wound. The remaining sets of cells were then placed in DMEM containing 0.1% CS for one day before being fixed. The ability of the cells to migrate into the wound was visualized by light microscopy and photographed.

Cell Growth Assays. Multiple sets of NIH3T3 cells stably expressing the vector alone or a Myc-tagged form of tTG were plated in 6-well dishes at a density of 2×10^4 cells/dish and maintained

in DMEM containing 0.1% CS. One set of the cells was counted every two days, while the medium on the remaining sets of cells was replenished. The growth assays were carried-out over a span of 6 days.

Apoptotic Assays. NIH3T3 cells stably expressing either the vector alone or a Myc-tagged form of tTG were seeded in 6-well dishes and then maintained in serum-free medium without or with PP2, LY294002, or rapamycin for 36 hours. The cells (both floating and attached) were then collected and stained with DAPI (2 μg/mL) for viewing by fluorescence microscopy. Apoptotic cells were identified by condensed and/or blebbed nuclei.

Immunoprecipitations. Cell lysates (typically 1.2 mg) were initially pre-cleared using protein G agarose beads. The pre-cleared lysates were then incubated with HA antibody for 1.5 hours, followed by the addition of protein G agarose beads for another 1.5 hours. After extensive washing of the beads with cell lysis buffer, Laemmli sample buffer was added to the beads and the samples were boiled.

Immunoblot Analysis. Whole cell lysates or the resulting immunoprecipitations that were performed were resolved by SDS-PAGE and the proteins were transferred to PVDF membranes. The membranes were incubated in primary antibodies prepared in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20. Horseradish-peroxidase conjugated secondary antibodies were used to detect the primary antibodies, followed by exposure to ECL reagent.

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CHAPTER 4

Conclusions

The localization of tTG at leading edges is important for cancer cell migration

The second chapter of this thesis described our findings regarding the importance of localizing tTG to leading edges for cancer cell migration. In this study, we determined that a small fraction of tTG (~10%) is constitutively associated with the plasma membrane and is activated and recruited to the leading edges of HeLa cervical carcinoma cells upon EGF stimulation. Given that tTG has no obvious membrane-interaction motif in its sequence, we hypothesized that a protein may be binding to tTG to facilitate its re-distribution along the leading edges in response to EGF. Immunoprecipitations of tTG from the membrane fractions of HeLa cells treated with EGF, led to the discovery that tTG binds the heat shock protein (Hsp)70 family of molecular chaperones. Both tTG and Hsp70 co-localize to leading edges and this localization is dependent on the chaperonin activity of Hsp70, as inhibitors against this function, prevent Hsp70 and tTG from localizing to leading edges. Importantly, these inhibitors also block the EGF-dependent migration of HeLa cells and the constitutive migration of MDA-MB231 cells, suggesting that the ability of tTG and Hsp70 to localize to leading edges is critical for cell motility.

These findings raise important questions regarding the nature of the relationship between tTG and Hsp70. Is tTG a client of Hsp70? Heat shock chaperones have specific client proteins and the expression, folding, or activation of these clients may be regulated by their respective chaperone (1,2). Inhibitors which block the function of heat shock proteins often lead to

decreased expression of these clients, thus many researchers have used this outcome as an indicator of chaperone-client interactions. In our experiments, no decrease in tTG expression was observed upon inhibition of Hsp70, suggesting that tTG is not a conventional client of Hsp70. However, given that these inhibitors do have an effect on the localization of Hsp70 and tTG to leading edges, this would suggest that an unknown client protein is involved in this regulation of tTG's sub-cellular localization. One attractive candidate for this client protein is c-Jun N-terminal kinase (JNK), given that Ras signaling through JNK was shown to regulate tTG activity and localization to leading edges. If Hsp70 were capable of regulating JNK expression and/or activation, this could help explain how Hsp70 promotes tTG localization to leading edges. While there is currently no evidence to support this hypothesis, the identification of this client is an important aspect for shedding light on the regulation of tTG.

These studies have also illustrated a novel role for heat shock proteins, namely to regulate the sub-cellular localization of a protein (tTG) to promote its role in cell migration. Heat shock proteins are generally classified as molecular chaperones, helping denatured proteins re-fold, or in some cases, helping to target these proteins for degradation by the proteosome when they are unable to be re-folded (1,2). However, in recent years researchers have discovered that these chaperones may help activate certain clients, in particular various signaling proteins such as Src kinase or B-Raf, and allow them to persist in an activated state even when they should be inactivated or degraded (2,3). As a result, there is much interest in heat shock proteins as a potential therapeutic target for the treatment of human cancer where aberrant cell signaling often contributes to malignant transformation (1,4,5). The finding that Hsp70 regulates the localization of tTG to leading edges, suggests that heat shock proteins may influence oncogenesis by helping proteins localize properly in order to carry-out their functions.

While we know that the ability of Hsp70 and tTG to localize to leading edges is important for the EGF-dependent migration of HeLa cells and the constitutive migration of MDA-MB231 cells, what tTG does at the leading edge to enhance motility is still unclear. Our initial hypothesis was that tTG may encounter a certain substrate at the leading edge and cross-link it to promote cell migration. One such substrate could be actin, as tTG has been shown to bind and cross-link actin *in vitro* (6). However, future studies will be necessary to determine the substrate(s) of tTG that may reside at the leading edge and whether cross-linking of this substrate(s) is important for tTG's ability to drive cell migration. To do this, one could isolate the membrane fractions of HeLa cells stimulated with EGF and perform tTG transamidation activity assays whereby the lysates will be incubated with calcium and a biotin-labeled substrate, i.e. biotinylated pentylamine (BPA). The crosslinked proteins can be purified using an affinity column with a resin of monomeric avidin and identified by mass spectrometry. Following identification of potential tTG substrates, the next step would involve knocking-down these proteins to determine if cell migration is impacted. Should these experiments lead to the discovery of a physiologically relevant tTG substrate important for cell migration, one could go on to generate a mutant form of this protein that cannot be crosslinked by tTG and determine whether crosslinking is critical for the ability of this substrate to promote cell migration.

However, in light of our second study (Chapter 3), the alternative possibility is that tTG regulates signaling events which may impact cell migration. Indeed, signaling proteins such as the EGF receptor and PI3-kinase have been shown to localize to leading edges (7). In this way, the leading edge may not simply be a structure for physically directing migration, but could also serve as a signaling hub where various proteins interact, leading to the activation of signaling networks which facilitate cell movement.

tTG plays a fundamental role in promoting cell survival

While there had been many studies describing the importance of tTG in cancer progression, to what degree tTG cooperated with oncogenic proteins in these cancer cell contexts, versus its intrinsic ability to promote malignant transformation was unknown. With this in mind, we set out to determine how the ectopic expression of tTG in a normal (non-transformed) cellular background would influence the behavior of the cells. Using NIH3T3 fibroblasts stably expressing the vector alone or a Myc-tagged form of tTG, we found that tTG strongly protected these cells from serum-starvation-induced apoptosis by activating the PI3-kinase/mTOR/p70 S6-kinase pathway. Moreover, tTG formed a 'signaling complex' with the non-receptor tyrosine kinase c-Src and PI3-kinase, which was important for the activation of the PI3-kinase pathway leading to enhanced cell survival.

While the evidence suggests that tTG has an inherent role in promoting cell survival, much remains to be learned about the interactions between tTG, c-Src, and PI3-kinase and how the formation of this signaling complex really leads to the enhanced signaling and survival observed in the fibroblast system. Some of the more obvious lines of study could involve determining which region of tTG binds to c-Src and PI3-kinase and testing to see whether mutations can disrupt these interactions. The best scenario would be to generate a tTG mutant incapable of binding either Src or PI3-kinase, and test this mutant to see whether the enhanced signaling and survival advantage is lost. This would provide more detail as to how this complex forms and confirm the importance of this complex in the signaling and survival outcomes.

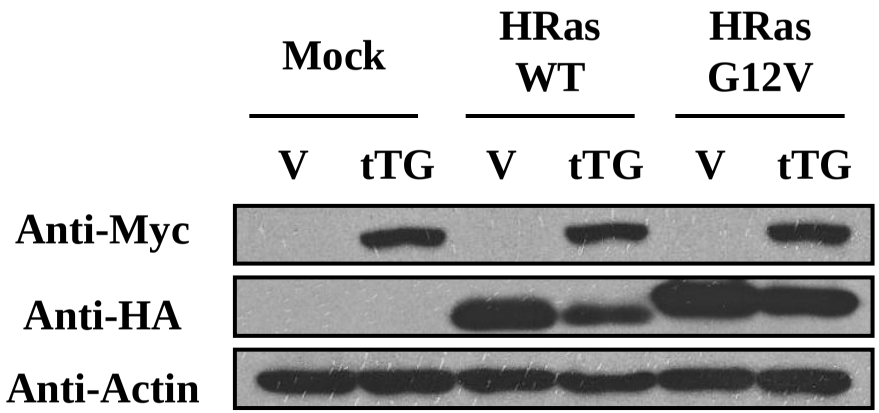
In addition, this mechanism needs to be evaluated in the context of human cancer cells. Of particular interest would be those cancer cells known to have elevated activation of Src or PI3-kinase, without direct mutation of either of these signaling proteins. Preliminary studies

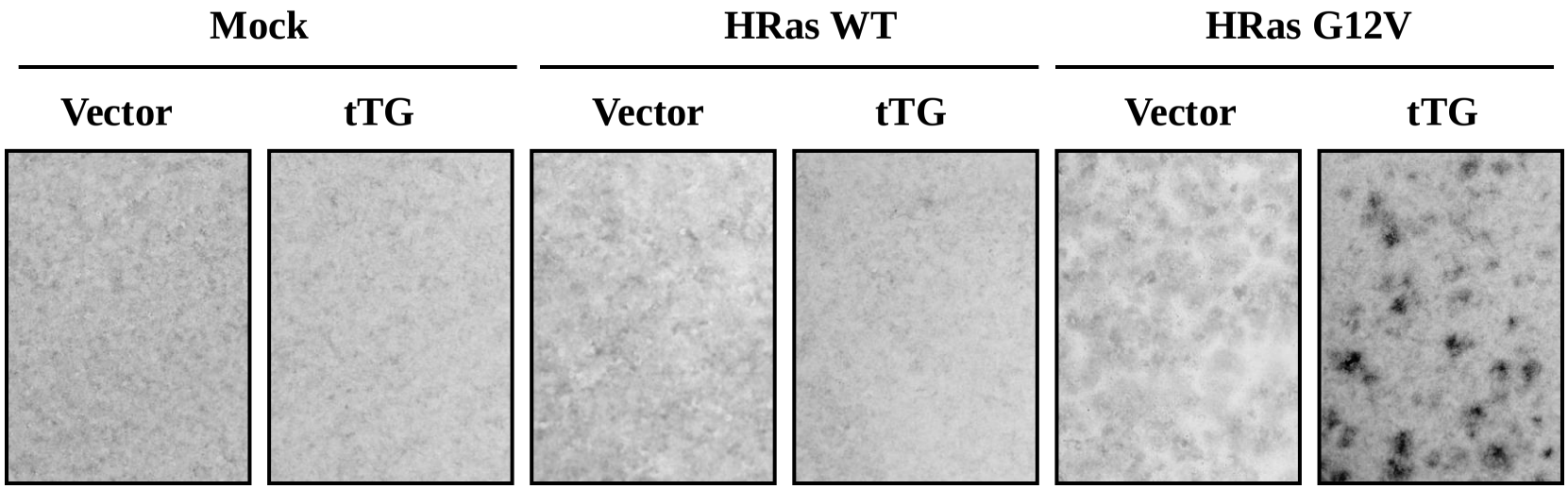
using the lung carcinoma cell line, A549, have supported our findings in fibroblasts. These cells express high levels of TG and show activation of the PI3-kinase/mTOR/p70 S6-kinase pathway. Treatment of the cells with inhibitors against tTG function (MDC) or Src activity (PP2), blocks the activation of this pathway and significantly inhibits transformation as read-out by reduced colony formation in soft agar assays. Determining which cancer types or cell lines utilize this mechanism will be important for understanding how tTG can globally impact cancer progression.

The role of tTG in Ras-driven cellular transformation

Given that our studies have shown that tTG inherently functions to promote cell survival through a Src-PI3-kinase dependent pathway, an important question concerns how tTG cooperates with oncogenes, such as Ras to drive oncogenesis. Ras is one of the most commonly mutated genes in human cancer, with a mutation frequency of ~30% in all types of cancer (8,9). Our early studies on the role of tTG in EGF-dependent migration showed that the EGF receptor signaled through Ras to direct changes in tTG activity and localization, suggesting that Ras and tTG may work together to promote cancer cell migration. This was determined using HeLa cells stably expressing the vector alone or an activated form of H-Ras (H-Ras G12V), whereby tTG was constitutively activated and localized to leading edges in the activated Ras-expressing cells compared to those expressing the vector alone. At this same time, we decided to analyze the role of tTG in Ras-driven transformation by performing soft agar assays without or with a tTG inhibitor (MDC). As expected, the cells stably expressing activated Ras formed larger and more numerous colonies compared to the vector-alone expressing cells. However, MDC treatment of the Ras-expressing cells led to a significant reduction in colony formation, suggesting that tTG was important for the transforming potential of Ras.

Figure 4.1 tTG synergizes with activated Ras to form foci. Cultures of NIH3T3 cells stably expressing the vector alone or a Myc-tagged form of wild-type tTG were transfected without (Mock) or with HA-tagged forms of wild-type (H-Ras WT) or activated Ras (H-Ras G12V). One set of cultures was lysed and subjected to Western blot analysis using the indicated antibodies. The other cultures were maintained in DMEM supplemented with 10% calf serum and 0.5 μg/mL puromycin for 10 days. The cells were then fixed with 3.7% formaldehyde and stained with 0.4% crystal violet to indicate differences in foci.


Mock
HRas WT
HRas G12V
V
tTG
V
tTG
V
tTG
Anti-Myc
Anti-HA
Anti-Actin

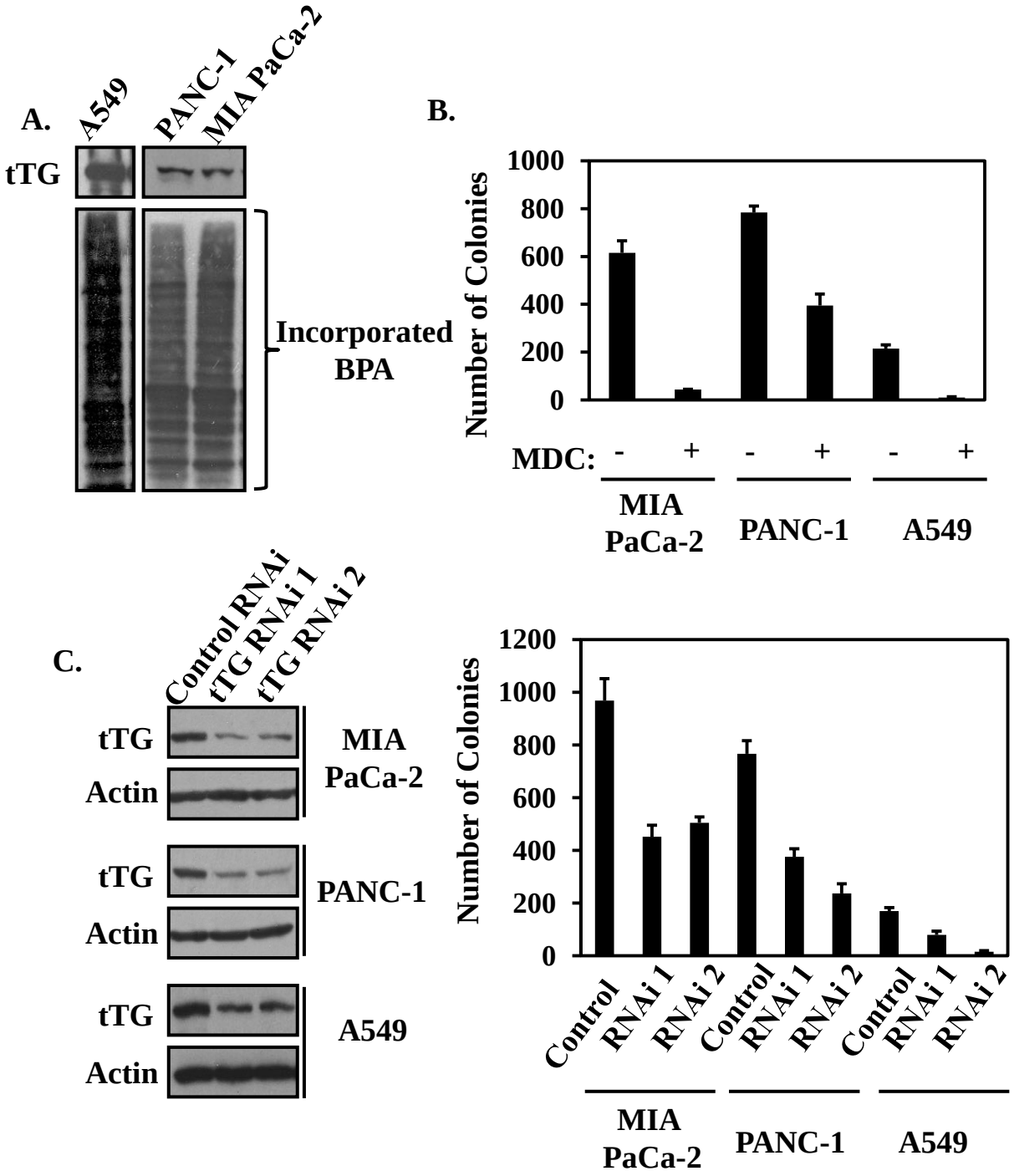

Mock
HRas WT
HRas G12V
Vector
tTG
Vector
tTG
Vector
tTG

With this in mind, we decided to read-out the impact of tTG on Ras-driven focus formation in another model system, the NIH3T3 fibroblasts stably expressing the vector alone or a Myc-tagged form of tTG. Expression of an activated form of H-Ras (H-Ras G12V) has been shown to induce transformation in several fibroblast cell lines by allowing the cells to overcome cell contact inhibition and form foci (i.e. areas of high cell density) (10). Interestingly, when Ras was ectopically expressed in the NIH3T3 fibroblasts stably expressing the vector alone or Myc-tagged tTG, we observed that expression of Ras in the tTG-expressing fibroblasts led to a much greater number of foci compared to expression of Ras in the vector-alone expressing fibroblasts (Figure 4.1). Thus, in this model system, Ras and tTG were able to synergize and enhance transformation.

We next examined the importance of tTG in human cancer cells known to possess mutations in K-Ras, including A549 lung carcinoma cells, MiA PaCa-2 and PANC-1 pancreatic cancer cells. Figure 4.2A shows that in these cell lines, tTG is highly expressed and activated. More importantly, tTG is required for the transforming ability of these cells as inhibiting its transamidation activity using MDC or knocking-down tTG using siRNAs significantly blocks the ability of the cells to form colonies in soft agar (Figures 4.2B and 4.2C, respectively). These findings suggest that tTG may play an important role in promoting Ras-driven transformation. We are currently investigating the role of tTG in these cell lines, specifically to see whether tTG works with Src and PI3-kinase to synergize with Ras and influence cellular transformation, as highlighted in Figure 4.3.

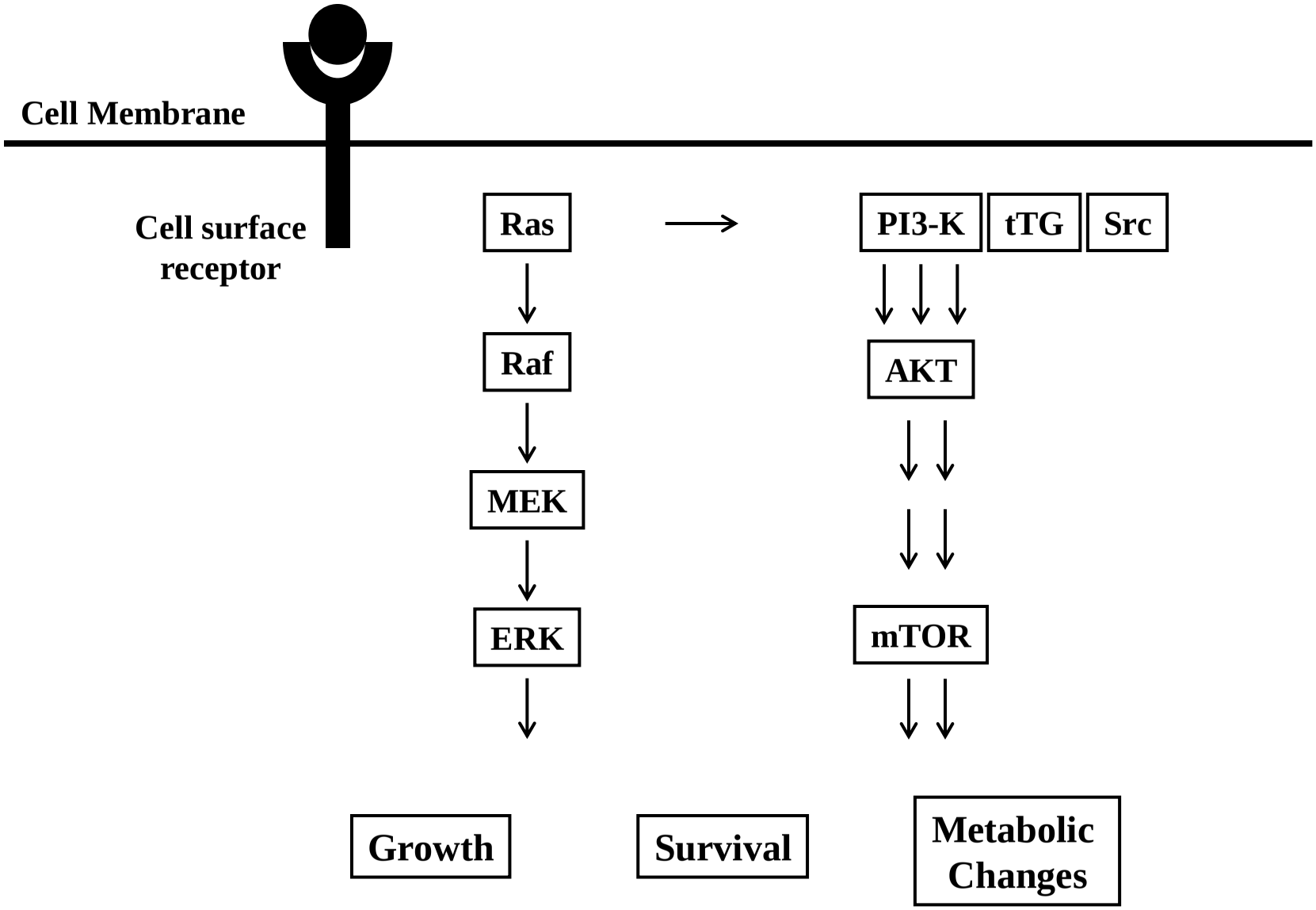
Overall these findings point to tTG playing a critical role in cellular transformation, influencing both cell survival and motility. They also suggest that tTG may be an important candidate for the development of therapies to target cancer cells. Future studies into the efficacy

Figure 4.2 K-Ras transformation is dependent on tTG. (A) A549, MIA PaCa-2, and PANC-1 cells were maintained in serum-free medium for 24 hours, lysed, and subjected to Western blot analysis. The same lysates were also assayed for tTG transamidation activity by determining the incorporation of biotinylated pentylamine (BPA) into lysate proteins. **(B)** Anchorage independent growth assays were performed on A549, MIA PaCa-2, and PANC-1 cells treated without or with MDC. Three separate assays were performed and the results were averaged. The error bars indicate the standard deviation. **(C)** Cultures of A549, MIA PaCa-2, and PANC-1 cells were transfected with control or two different tTG siRNAs. One set of cultures was subjected to Western blot analysis (*left panels*), while the remaining lysates were used to perform anchorage independent growth assays (*right panel*). Three separate assays were performed and the results were averaged. The error bars indicate the standard deviation.


A.
A549
PANC-1
MIA PaCa-2
tTG
Incorporated BPA
B.
Number of Colonies
1000
800
600
400
200
0
MDC: - + - + - +
MIA PaCa-2
PANC-1
A549
C.
Control RNAi
tTG RNAi 1
tTG RNAi 2
tTG
Actin
MIA PaCa-2
PANC-1
A549
Number of Colonies
1200
1000
800
600
400
200
0
Control
RNAi 1
RNAi 2
MIA PaCa-2
PANC-1
A549

of targeting tTG will be very valuable. Though there is the possibility that tTG may play some undiscovered role in normal development and may not be the best candidate for therapeutic intervention, there is a growing amount of evidence which contradicts this idea and favors the hypothesis that tTG is a worthwhile target.

Figure 4.3 tTG may influence Ras transformation by activating Src and PI3-kinase. Based on our previous findings that tTG can bind Src and PI3-kinase in NIH3T3 mouse fibroblasts and induce the activation of the PI3-kinase signaling to promote cell survival, it will be important to determine if the same mechanism applies in the context of Ras-driven cancer cells.


Cell Membrane
Cell surface receptor
Ras
Raf
MEK
ERK
PI3-K
tTG
Src
AKT
mTOR
Growth
Survival
Metabolic Changes

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